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INVESTIGATIONS OF HEAT-LABILE AND HEAT-STABLE ENTEROTOXINS PRODUCED BY *ESCHERICHIA COLI* ISOLATED FROM NATURALLY OCCURRING BOVINE COLIBACILLOSIS

BY

JUDY CAROL KIENHOLZ

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Microbiology, South Dakota
State University
1976

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INVESTIGATIONS OF HEAT-LABILE AND HEAT-STABLE ENTEROTOXINS
PRODUCED BY *ESCHERICHIA COLI* ISOLATED FROM
NATURALLY OCCURRING BOVINE COLIBACILLOSIS

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Advisor   Date

Head, Department of Microbiology   Date

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JCK

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INTRODUCTION

Colibacillosis (CB) is a collective term which has been used to describe a group of diseases caused by the gram negative bacillus *Escherichia coli*. The disease affects neonatal calves, lambs, and piglets, weaned pigs, older pigs and poultry. Certain *E. coli* also cause a diarrheal disease in human adults and infants (1).

Escherichia coli have been divided into two groups, pathogenic and non-pathogenic. The pathogenic strains may be further divided according to the manifestations of the infection. The differences are in tissue localization of the *E. coli* and in the biological activity of the *E. coli* enterotoxins responsible for each syndrome. Therefore, the enteric diseases caused by *E. coli* are described as enterotoxic, enterotoxemic and local invasive CB. The characteristic features of enterotoxic CB are proliferation of *E. coli* in the lumen of the small intestine with no invasion of the epithelial cells (39, 41) and production of an enterotoxin which causes the small intestine to secrete abnormally large volumes of fluid. Enterotoxemic CB manifests itself as localization of *E. coli* in the small intestine where the bacteria produce a toxin which is absorbed and acts elsewhere. Edema disease in swine and entericitoxemic CB in calves (16) are two well-known examples of this type. Invasion and destruction of intestinal epithelium by *E. coli* are the characteristics of local-invasive CB. The host develops ulcerative enteritis, dysentery and fever. Local invasive colibacillosis is similar to shigellosis (38). The work reported herein will be concerned only with the enterotoxic form of CB and the enterotoxins produced by *E. coli*.

Twenty-five percent of the hog deaths before market are due to CB. In South Dakota alone this is about a $5,000,000 annual loss (59). Enterotoxic CB often affects over 50% of the newborn calves in a herd and death rates of 10 to 20% are common (46). The disease is characterized by a profuse watery diarrhea, severe dehydration, electrolyte imbalance, apathy, hypothermia and death if therapeutic measures are not taken. When the entire herd is affected, losses to the producer can be extremely high due to death and unthriftiness of animals which survive.

Two types of *E. coli* enterotoxin have been described. Both may be important in enteric disease of humans and animals. One toxin is of low molecular weight, is heat stable, acid stable, dialysable, and is not antigenic (7). The other toxin is of high molecular weight, acid sensitive, not dialysable and is very similar to *Vibrio cholerae* enterotoxin in its heat lability, intestinal response, and it is related immunologically to cholera toxin. The heat stable toxin (ST) can be detected by gut loop ligations and suckling mouse assays whereas gut loop ligations and cytopathic effects in tissue culture are used to detect heat labile toxin (LT).

Most of the enterotoxin purification research has been done on the LT, however, both enterotoxins must be purified and characterized before the pathogenesis of enteric colibacillosis can be fully elucidated.

The research reported herein includes examination of 37 bovine *E. coli* strains to determine entero pathogenicity and the reliability of different assay methods, characterization of bovine LT and comparison of bovine LT with porcine LT, comparison of three culture media to determine which one provides maximum ST production, and partial purification of bovine ST.

LITERATURE REVIEW

Escherichia coli was first associated with disease by Jensen (26). The disease was calf diarrhea which he stated had existed in Denmark for 100 years. He was able to distinguish between pathogenic and nonpathogenic strains of E. coli by the oral administration of live cultures to newborn calves. The pathogenic strain caused a disease similar to that observed in calves infected naturally, while the nonpathogenic strain did not.

In the 1920's, Theobald Smith of the U.S. co-authored several papers relating to calf diarrhea. He and his colleagues confirmed the importance of E. coli in "white scours" of calves and the importance of colostrum in maintaining an equilibrium between host and pathogenic organism (58). It wasn't until the late 20's and early 30's that E. coli was associated with neonatal diseases of animals other than calves. In 1927 Adam associated it with human infantile gastroenteritis (1).

Efforts by T. Smith et al. to distinguish pathogenic strains from nonpathogenic strains by biochemical differences were unsuccessful (57). Lovell (36) was the first to serologically identify calf strains of E. coli. He accomplished the differentiation by using precipitin tests. Goldschmidt (18) attempted to apply serologic methods for the identification of enteropathogenic E. coli (EEC) which caused human infantile gastroenteritis. He was able to apply his serologic methods to epidemiologic studies of human infantile gastroenteritis. Kauffman (29) used the serologic techniques which had been developed for Salmonellae on E. coli. This allowed for differentiation between different types

of *E. coli*. It was then possible to compare strains of *E. coli* from animals with various disease syndromes throughout the world.

Stevens (60) stated that piglets infected with colibacillosis (CB) in the first few days of life may show diarrhea, listlessness, dehydration and death associated with the recovery of large numbers of a pure culture of a certain strain of *E. coli* from the intestinal tract. The newborn pig is predisposed to CB during the first few days of life because it is at this time that the small intestine is most susceptible to colonization by *E. coli* (40). This is due to a combination of factors, such as the high pH of the stomach at birth which does not kill the organisms ingested, and the decreased intestinal motility soon after birth which allows the bacteria to colonize without being "flushed out" by normal peristalsis. Also, there is no competition between normal flora and the pathogenic organisms because no flora has been established. As the pig becomes older, the pH lowers, peristalsis increases and a normal flora is eatablished. Thus the intestine becomes less sensitive to infection.

Although it was generally accepted that *E. coli* caused a severe diarrhea in neonatal animals, the pathogenesis of the disease was poorly understood. Several studies were initiated so that the pathogenesis could be defined. Smith and Jones, (53) in 1963, fed live cultures of *E. coli* to newborn pigs and produced diarrhea. Diarrhea in human adults and infants could also be produced with *E. coli* if the numbers of organisms ingested were large enough (13). Recognition of *E. coli* as a possible pathogen accounted for many previously undiagnosed cases of

human diarrhea (15). Kohler (33) was able to produce neonatal diarrhea in gnotobiotic pigs by the oral administration of live E. coli. A parallel method of investigating the pathogenic effects of E. coli made use of the ligated rabbit gut loop which was injected with living (4, 62) or chloroform-killed (61) E. coli cultures. Fluid accumulation and dilatation of the loops following the intraluminal injection of test materials was considered a positive reaction (32).

The ligated intestinal segment (LIS) test was used by Smith and Halls (50) on porcine, ovine, bovine and avian gut to study strains of E. coli from domestic animals. To obtain evidence of the validity of the test, the same strains used in loops were given orally to intact animals in order to determine whether or not they would produce diarrhea. These researchers found good correlation between the reaction of an E. coli strain that produced dilatation in a ligated segment of the small intestine and ability of the strain to produce diarrhea. They went on using the LIS with cell-free preparations (51) instead of live cultures to produce dilatation. Bacteria-free filtrates had previously been utilized in Vibrio cholerae toxin research (5).

Smith and Halls stated that to be able to produce diarrhea, a particular strain of E. coli must possess at least two properties. It must be able to produce enterotoxin and it must be able to proliferate in the anterior small intestine (50). It was suggested that the massive flow of fluid into the small intestine which occurs in E. coli diarrhea is the response of the host to production of the enterotoxin

by the *E. coli* organisms proliferating in the anterior small intestine.

Endotoxin, which is present in all gram negative organisms, can produce diarrhea when injected intravenously. The heat stability, time of appearance in growing cultures, and dilatatory effects on LIS of enterotoxin distinguish enterotoxin from endotoxin (51). These factors indicate that endotoxin has no primary role in the pathogenesis of diarrhea caused by EEC (21, 31, 33, 50, 51, 63).

The enterotoxic activity in the cell-free filtrates used by Smith and Halls was heat stable (51) and controlled by plasmid transmission. A heat labile enterotoxin (LT), as well as a heat stable enterotoxin (ST), produced by EEC strains was described by Gyles (21). The ability to produce LT is also transferred by a plasmid.

Several tests, in addition to heat sensitivity, can be used to distinguish between ST and LT. The heat stable enterotoxin is of low molecular weight, between 1,000 and 10,000 (2,37), is apparently non-antigenic, is stable to 85 C (49), and its activity is resistant to acid, trypsin, and pronase (24). In the ligated loop of rabbit intestine, the onset of net accumulation of fluid in response to ST appears to be immediate, even at low doses (11, 45). Its mechanism of action in the intestine is not known.

The LT has a molecular weight of about 102,000 (8), is not dialysable, is antigenic, and is inactivated at 65 C for 30 minutes. Its activity is acid labile and sensitive to pronase which indicates that the enterotoxic activity resides in material of a protein nature (8, 12, 25, 21). Onset of net accumulation of fluid in response to LT is

rapid at high doses, but delayed at low doses (11, 45). Dorner (8) has purified LT by using gel filtration chromatography and preparative isotachophoresis. However, the enterotoxin has not been completely characterized.

The LT has some characteristics similar to the Vibrio cholerae enterotoxin. The E. coli LT is immunologically related to V. cholerae enterotoxin since it can be neutralized by V. cholerae antitoxin (20, 21, 45, 56). Its mechanism of action is also similar in that both stimulate adenylate cyclase activity in the epithelial cells of the small intestine (28, 9), turning on a cyclic adenosine 3', 5' monophosphate (cAMP) mediated fluid transport system to a pathologic degree (27), resulting in severe losses of water and electrolytes into the lumen of the small intestine which leads to severe diarrhea.

Various media and incubation methods have been used for enterotoxin production by E. coli. Soft agar medium was used by Smith and Halls (51) and Bywater (2) for production of ST. Kohler (31) used a syncase medium which Finkelstein (14) had previously used for production of V. cholerae enterotoxin. Peptone dialysate medium was used by Lariviere et al. (35) for ST and LT preparation. Tryptic Soy Broth (TSB) has been used widely for ST and LT production (8, 10, 42). Moon (43) demonstrated that agitation during incubation increased the number of cells and the amount of enterotoxin per cell. The use of Mitomycin C (23) in culture media has induced 8-96 fold increases in LT production compared to uninduced cultures. No chemical has been found to increase ST production.

The ligated intestinal loop technique has been utilized for most of the reported work with *E. coli* enterotoxins in domesticated animals (41, 50, 61, 62), however, the technique is expensive, time consuming, cumbersome, and allows considerable latitude for error. These features make the assay unsuitable for rapid identification of enteropathogenic *E. coli*. The results of serological investigations are not entirely accurate in assessing the enteropathogenicity of *E. coli* because the production of enterotoxin is not determined by a capsular or somatic antigen, but rather by the presence of the enterotoxin plasmid which can be transferred from one bacterium to another by conjugation (22). Several investigators have made use of morphological changes in tissue culture as a quick accurate assay for LT. Cat heart cells (8), adrenal tumor cells (7, 34, 48), Chinese hamster ovary cells (19), Hela cells (30), and pig thyroid cells (47) have been used. Intragastric inoculation of suckling mice resulting in fluid accumulation in the intestine has been demonstrated to be a quick, reliable method for detection of ST (6, 10).

Factors other than the production of enterotoxin by EEC are necessary to produce colibacillosis in animals. First, high numbers of the pathogenic organism must be present in the intestine to produce enough enterotoxin to elicit intestinal response. The organism must either be ingested in extremely large numbers or else they must proliferate in the intestine after being ingested. It has been found that in spontaneous enterotoxic CB, *E. coli* proliferated in the anterior small intestine to numbers from 10 to 10,000 times normal (53). Research

in reproduction of CB demonstrated that EEC colonized the small intestine more intensively than did nonenteropathogens (32, 50). Production of colicins, antibiotic-like substances produced by enteric bacteria, would presumably give a colonization advantage to the enteropathogen. However, investigation of this apparent advantage of colicin producers to colicin non-producers revealed that the production of colicin has a minimal effect on colonization (3). Enteropathogens seem to resist the effects of intestinal motility by adhering to the mucosa of the intestinal wall while nonenteropathogens lack this ability (50). This attachment allows the organism to proliferate next to the absorptive epithelial cells while nonenteropathogens only randomly come in contact with the epithelium. A surface structure antigen, designated as K88 antigen, confers adhesive properties to *E. coli* which possess the K88 antigen. This antigen is different from most known surface antigens of *E. coli*. It occurs as a fur of fine filaments extending well beyond the cell wall and is morphologically and chemically distinct from the fimbriae of *E. coli*. K88 antigen is a protein structure whereas other capsular antigens are polysaccharide. A similar K99 antigen (55) is found in some calf strains. Both of these antigens are coded by transmissible plasmids (54). There is evidence that pili can contribute to the adhesive and colonizing abilities of EEC which do not contain the K88 or K99 antigen (44).

MATERIALS AND METHODS

E. coli Strains

All E. coli strains used throughout this work were obtained from field cases of colibacillosis submitted to the Animal Disease Research and Diagnostic Laboratory (ADRDL), South Dakota State University, Brookings, South Dakota. The cultures were tested biochemically to confirm their identification and were serotyped according to methods described by Glantz (17). The cultures were stored in Tryptic Soy Agar stabs (Difco Laboratories, Detroit, MI) in the dark at room temperature.

Enterotoxin Production

Erlenmeyer flasks (250 ml) containing 10 ml sterile broth medium were inoculated with 5 ml of an overnight broth culture of E. coli. The flasks were closed with cotton stoppers and incubated 18 hours at 37 C while on a rotary shaker (225 rpm) (42). Following incubation, bacteria were removed by centrifugation at 9750 x g for 30 minutes at 4 C. The bacteria-free supernatant was withdrawn and stored at 4 C or at -70 C. These preparations were used for ST and LT assays, gut ligation studies, and ST purification studies.

Three media, tryptic soy broth without dextrose (Difco), peptone dialysate (35), and syncase (32), were compared in initial enterotoxin production studies. Tryptic soy broth without dextrose (TSB) was used for all further enterotoxin production.

Enterotoxin Assays

Ligated Intestinal Segments. Three- to five-week-old piglets of mixed breed were obtained from the SDSU Swine Unit. Two ten-day-old Holstein-Fresian bull calves were purchased locally. The animals were

fasted 24 hours prior to use. A Connel 201 Veterinary Anethesia Machine (Pitman-Moore, Inc., Minneapolis, MN) with Metofane (methoxy-flurane) was used to keep the animals anesthetized during the entire surgical procedure. Nine to 16 ligations (piglets), and 30-45 ligations (calves), 10 cm long, were placed in the small intestine starting 3 to 4 m posterior to the pyloric sphincter. Each loop was inoculated with 1 ml (piglets) or 3 ml (calves) enterotoxin. Uninoculated inter-loops, 3 cm long, were placed between each inoculated loop in order to control leakage from the inoculated loops. Eighteen to 24 hours later the animals were killed by the administration of large doses of sodium pentabarbital. The abdomen was opened immediately and the entire small intestine carefully removed and separated from its mesenteric attachment. The presence or absence of dilatation of the inoculated segment was recorded.

The positive control used in all ligation experiments was a supernatant from _E. coli_ strain 263 obtained from H.W. Moon (National Animal Disease Center (NADC), Ames, IA). Sterile TSB (Difco) was used as the negative control. If these control loops did not produce correct responses in the animal, the results of that particular animal were discarded.

Suckling Mouse Test. Four- to six-day-old infant Webster Swiss mice (approximately 3 grams) were separated from their mothers and randomly divided into groups of 5-7. The mice were inoculated with 0.1 ml of broth supernatant through the body wall directly into the milk-filled stomach with a 30 gauge hypodermic needle. Crystal violet did not

interfere with the test so one drop was added to each 1 ml of inoculum to aid in detecting proper injections.

After inoculation the mice were kept at room temperature (25 C) for four hours and then killed with chloroform. The abdomen was opened and the entire intestine removed. Mice with dye in the peritoneal cavity or bladder were discarded. The intestines and bodies were weighed separately and the ratio of intestinal weight to body weight was calculated. Ratios greater than 0.09 were considered positive and indicated the presence of ST in the broth supernatant. Those in the range of 0.0799 to 0.0899 were considered equivocal and the test was repeated at least twice. Ratios less than 0.0799 were considered negative.

Cell Culture Assay

Y1 mouse adrenal cells were obtained from Dr. H.W. Moon (NADC). The cells were grown in Ham F-10 (Gibco, Grand Island, NY) tissue culture medium supplemented with 20% Fetal Bovine Serum, 0.1 g/l Streptomycin, 0.1 g/l Kanamycin, and 0.08 g/l Penicillin. Each well of a 96 well tissue culture plate (MicroTest II, No. 3040 Falcon, Oxnard, CA) was inoculated with 0.025 ml of Y1 cell suspension sub-culture and 0.1 ml tissue culture medium. The inoculated plates were incubated at 37 C in an atmosphere containing 5% CO_2 and 100% humidity for approximately 48 hours or until confluent monolayers were obtained. One drop (0.05 ml) of broth supernatant was added to each well. A positive control (Strain 263, serotype 08:K87, 88:H19, NADC) and a negative control (Strain 431 serotype 0101:K(A):NM, NADC) enterotoxin

were included for each test plate. Following addition of enterotoxin, the plates were reincubated for 18-24 hours. Results were assayed by microscopic observation of the cellular monolayer. Marked rounding of the cells was considered positive for the presence of heat-labile enterotoxin.

Enterotoxin neutralization

E. coli LT antiserum was obtained from Dr. H.W. Moon (NADC). The antiserum was produced from porcine strain 72-2502 isolated from a field case of porcine colibacillosis submitted to ADRDL. The antiserum was diluted 1:100 with tissue culture medium (see above). Equal amounts of diluted antiserum and enterotoxin preparations of LT positive strains were mixed and incubated for 60 minutes at 37 C and then assayed for LT neutralization in calf intestine and cell culture.

Heat Inactivation

The enterotoxin preparations from LT producing bovine strains were heated in a water bath, one group at 65 C for 30 minutes, another group at 85 C for 30 minutes. The heated samples were assayed in Y1 cell culture for LT activity.

Protein analysis

Protein was estimated by the biuret method. Lyophilized human albumin in 0.9% NaCl (American Hospital Supply Corporation, Miami, FL) was used as the standard.

Extraction of ST with Methanol

The method described by Kohler (33) was used to extract ST from broth cultures. Cell-free supernatant (300 ml) was lyophilized and

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resuspended in 150 ml methanol. The suspension was shaken for an hour, 100 ml of extract poured off and 100 ml new methanol added and shaken one hour. The extract was again poured off and 50 ml methanol added. The extract and all solid material were filtered through a Buchner filter and evaporated to dryness with a Buchi Rotovapor flash evaporator (Arthur H. Thomas Co., Philadelphia, PA) in a 65 C water bath. The dry matter was resuspended in approximately 10 ml double distilled water and refrigerated for further purification.

Ultrafiltration (UF)

Cell-free supernatants of *E. coli* cultures were filter sterilized with a Seitz pressure filter. The supernatants were subjected to sequential UF through Amicon XM-100, XM-50, and UM-10 membranes (Amicon Corp., Lexington, MA) which filtered out molecular weights above 100,000, 50,000, and 10,000, respectively. Ultrafiltration was carried out at room temperature under nitrogen at 25-40 lbs/in^2 in an Amicon TCF-10 cell. Retentate was washed twice, tested for enterotoxicity, lyophilized, and reconstituted to 20 ml with sterile distilled water.

Gel filtration chromatography

A 2.5 x 95 cm column was packed with Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ) hydrated in 0.02 M NaCl with 0.02% NaN_3 added. Methanol extracts or ultrafiltered concentrates were applied in volumes of 3-20 mls and eluted with 0.02 M NaCl and 0.02% NaN_3. Fractions were collected in 6.4 ml portions at a flow rate of approximately 64 ml/hour. The effluent from the columns was continuously monitored for ultraviolet-light absorption at 280 nm

using a Gilford automatic recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). Fractions containing UV-absorbance were pooled, lyophilized and reconstituted in 10 ml sterile distilled water.

Ion Exchange Chromatography

A 440 mg portion of the Sephadex G-25 ST-active peak dissolved in 0.05 M phosphate buffer was placed on a column (2 x 10 cm) containing Sephadex A-50 (Pharmacia Fine Chemical) equilibrated with 0.05 M phosphate buffer at pH 6.3. The sample was eluted at a flow rate of 45 ml/hour, beginning with the phosphate buffer and proceeding with a step-wise addition of 0.01 M, 0.02 M, 0.03 M, 0.05 M, 0.075 M, and 0.10 M NaCl Fractions were collected in 3.2 ml volumes with at least 50 ml collected for each buffer-NaCl concentration. The eluent was monitored at 280 nm and fractions comprising each of the peaks were pooled and tested in suckling mice for ST activity.

RESULTS

Examination of bovine *E. coli* strains

Thirty-seven *E. coli* strains isolated from naturally occurring cases of calf colibacillosis were assayed in suckling mice (Figure 1,2), Y1 adrenal cell culture (Figure 3, 4), porcine LIS (Figure 5), and bovine LIS (Figure 6) to determine whether the strains were enterotoxigenic. The strains, their hemolysis, and their enterotoxic responses to assay methods are listed in Table 1. Seventeen enterotoxin preparations showed no enterotoxic responses in any of the enterotoxin assays. Seven preparations showed ST activity in mice, but were negative in LT and LIS assays. Eight preparations showed LT activity in cell culture and five of these also caused distention due to fluid accumulation in bovine LIS. One strain produced both ST and LT, showing enterotoxic activity in all assays.

Heat- and antiserum-inactivation of LT

LT activity of all strains was inactivated at 85 C/30 minutes. At 65 C/30 minutes two enterotoxin preparations still retained some LT activity. Rabbit anti-porcine LT completely inactivated the bovine LT in the Y1 adrenal cell culture assay. In the calf LIS half of the LT preparations, following incubation with rabbit anti-LT, caused fluid accumulation in the test loops. The results are listed in Table 2.

Media comparison

Three *E. coli* ST producing strains, 74-3717 (0138:NM), 74-656 (09:NM), and 74-2508 (020:NM), were each grown in three different

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3 15

Figure 1. Appearance of suckling mice killed 4 hours after inoculation of tryptic soy broth (upper mouse, negative control) or ST (lower mouse, positive control). Note the distension of the lower mouse intestine caused by excess fluid accumulation due to activity of ST.

Figure 2. Appearance of suckling mouse intestine removed 4 hours after inoculation of tryptic soy broth (left) or ST (right).

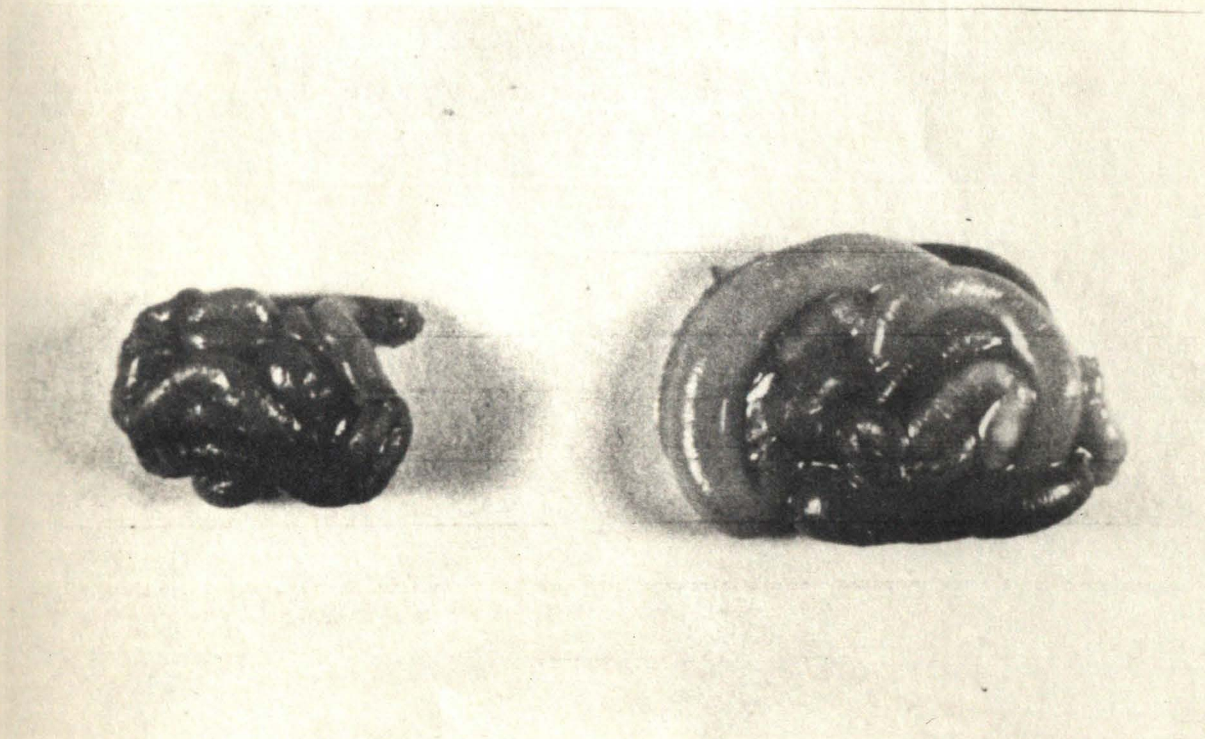
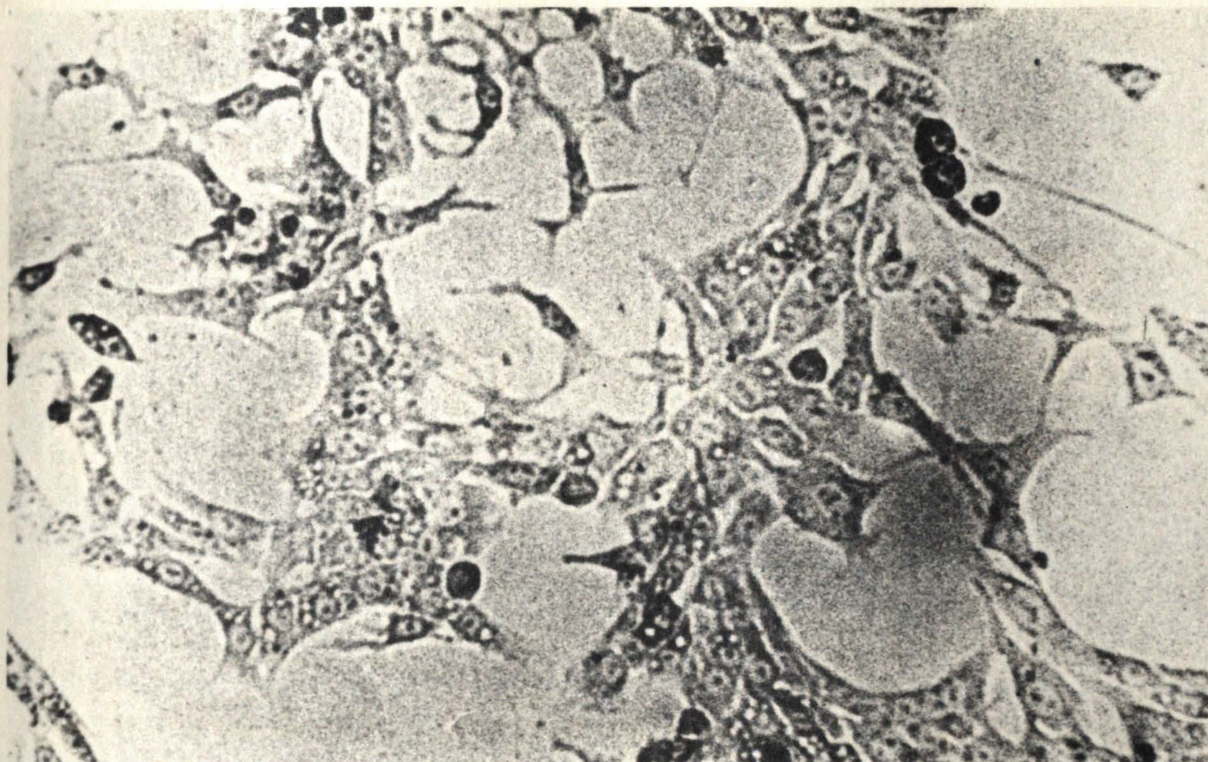


Figure 3. Appearance of normal cell layer of Y1 mouse adrenal tissue culture cells.

Figure 4. Appearance of Y1 mouse adrenal cells 18 hours after inoculation with LT. Note the rounding of most cells which was not present in normal cell layer. Marked rounding of Y1 adrenal cells is indicative of LT activity.



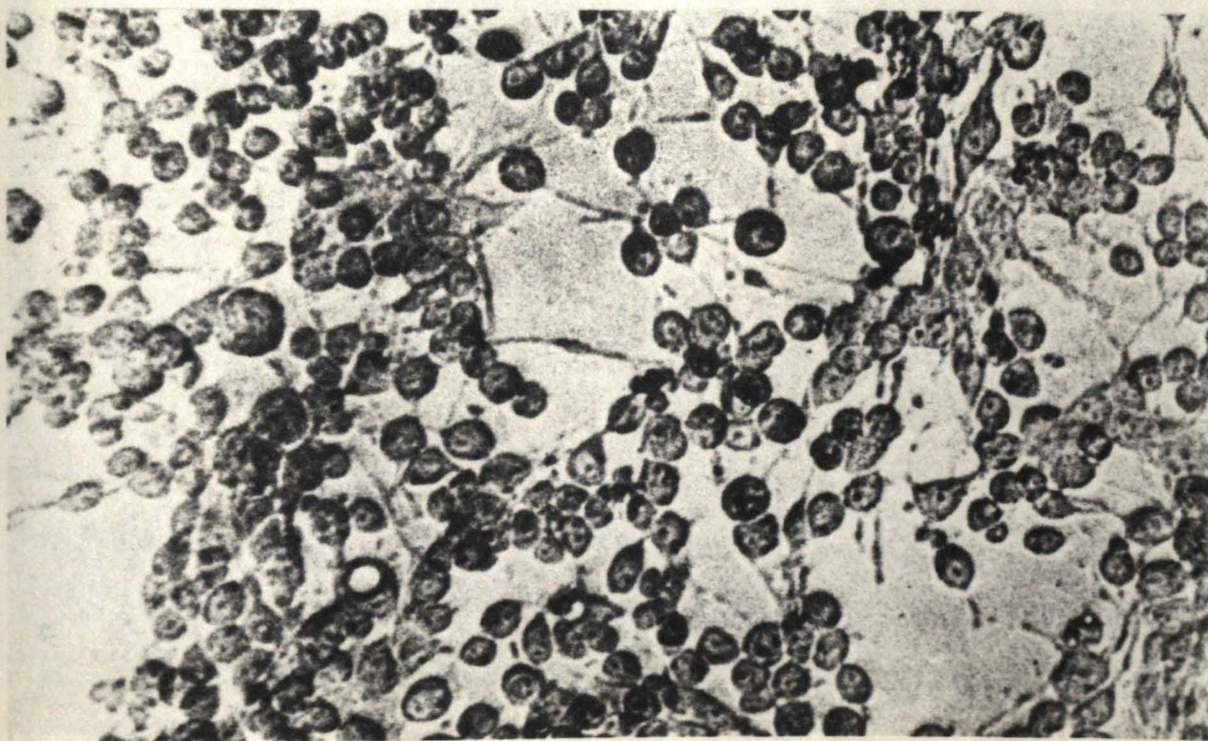


Figure 5. Appearance of porcine ligated intestinal segments 18 hours after inoculation with enterotoxin preparations. Inoculated loops (10 cm) were separated by uninoculated interloops (3 cm). In both intestines, enterotoxins produced by strains 73-3752 and 263 (positive control) were the only segments which were distended by fluid accumulation due to enterotoxic activity. The others were considered negative.

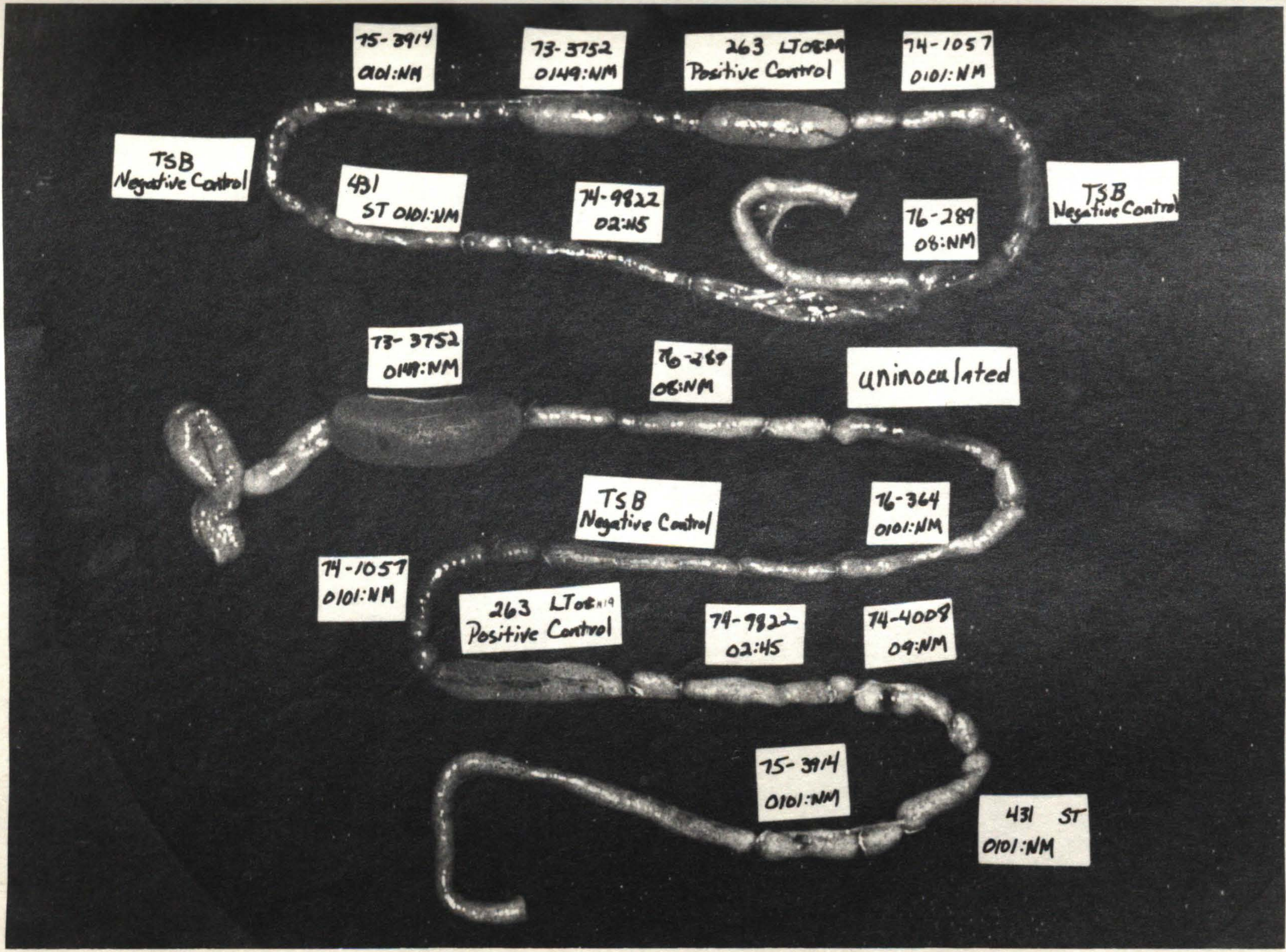

75-3914 0101:NM
73-3752 0149:NM
263 LT Positive Control
74-1057 0101:NM
TSB Negative Control
431 ST 0101:NM
74-9822 02:H5
76-289 08:NM
TSB Negative Control
73-3752 0149:NM
76-289 08:NM
uninoculated
TSB Negative Control
76-364 0101:NM
74-1057 0101:NM
263 LT Positive Control
74-9822 02:H5
74-4008 09:NM
75-3914 0101:NM
431 ST 0101:NM

Figure 6. Appearance of bovine ligated intestinal segments 18 hours after inoculation with enterotoxin preparations. Inoculated loops (10 cm) were separated by uninoculated interloops (3 cm). Loops 75-3140, 76-364, 74-3256, 75-2003, 74-9822, 73-3752, 75-3914, 75-750Lg, and 73-1929 were considered positive. Note the distention in the positive loops due to fluid accumulation caused by enterotoxin activity.

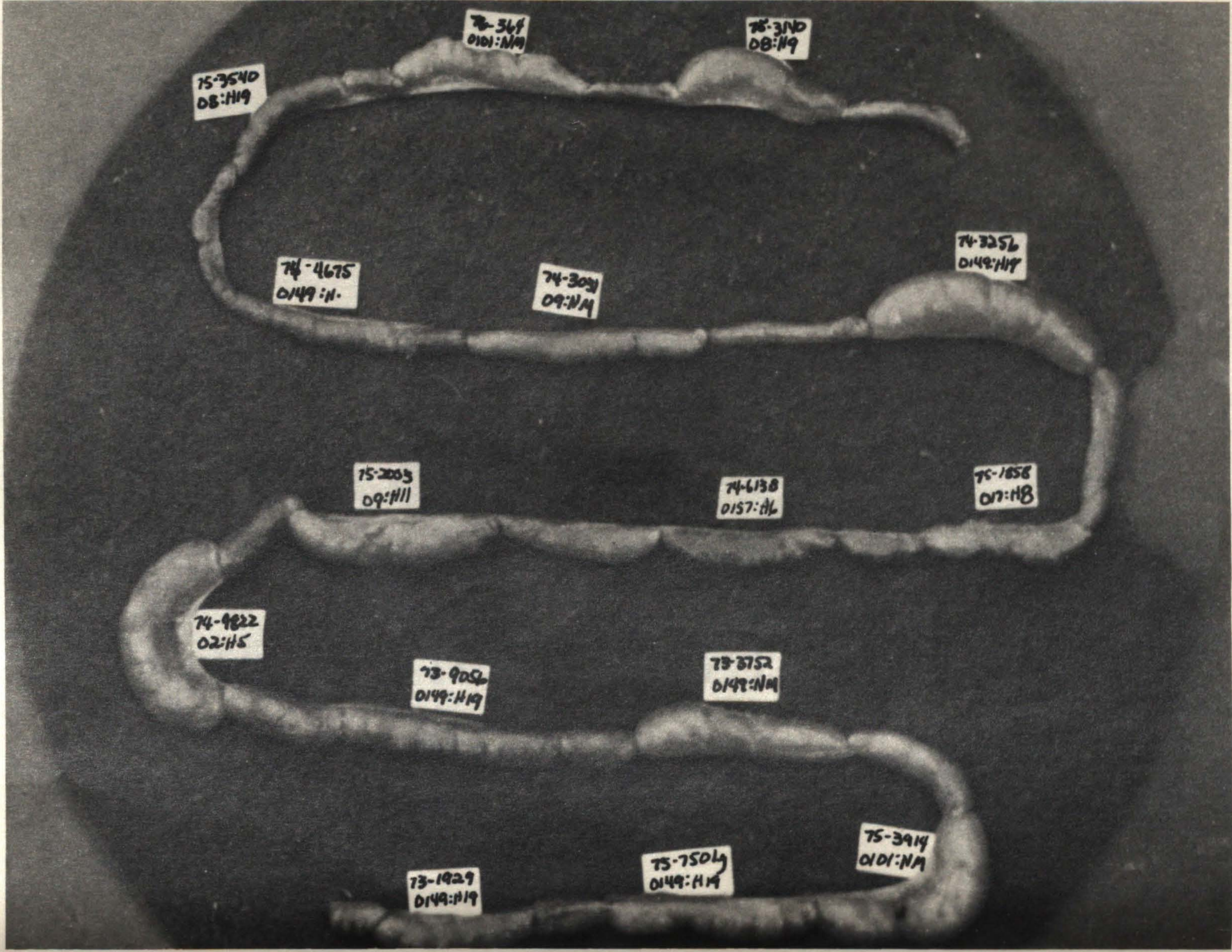

75-3540
08:H19
74-4675
74-3256
0149:H19
75-2003
09:H11
75-1858
017:H8
74-9822
02:H5
73-9056
0149:H19
75-3914
0101:NM
75-7504
0149:H19
73-1929
0149:H19

Table 1. Production of enterotoxin and hemolysis by *E. coli* isolated from natural occurring cases of bovine colibacillosis

Isolate	Serotype	Hemo-lysis[a]	Enterotoxin Assays				
			Mouse Assay (ST)	Cell Culture (LT)	Pig loop	Calf 1	Calf 2
74-9822	02:H5	H[b]	-	-	-	+	-
75-3346	08:H9	H	-	-	-	NT[c]	-
75-3540	08:H19	NH[d]	-	-	-	-	-
75-3764	08:H19	NH	-	-	-	NT	-
75-2003	09:H11	NH	-	-	-	+	-
74-3031	09:NM	NH	-	-	-	-	-
75-1858	017:H8	NH	-	-	-	-	-
75-X77	017:H8	NH	-	-	-	NT	-
75-9661	026:H6	NH	-	-	-	NT	-
76-251811S	075:H2	NH	-	-	-	+	-
74-2133	075:H2	H	-	-	-	NT	-
74-4101	075:H2	H	-	-	-	-	-
72-3345	0138:H-	NH	-	-	-	+	-
76-5163-86	0139:H-	H	-	-	-	-	-
76-25189R	0149:H2	NH	-	-	-	-	-
73-4588	0149:NM	NH	-	-	-	-	-
73-6209	0149:H-	NH	-	-	-	-	-
74-4675	0149:H-	NH	-	-	-	-	-
74-4881	0149:NM	NH	-	-	-	-	-
74-613	0157:H6	NH	-	-	-	-	-
74-1657	0157:H10	NH	-	-	-	-	-
76-289	08:NM	NH	+	-	-	+	-
75-3140	08:H9	H	+	-	-	+	+
74-4008	09:NM	NH	+	-	-	+	-
76-364	0101:NM	NH	+	-	-	+	+
75-3914	0101:NM	NH	+	-	-	+	+
74-4498	0101:NM	NH	+	-	-	+	-
74-1057	0101:NM	NH	+	-	-	+	+
73-1611	0149:H19	H	-	+	-	+	+
73-3738	0149:H19	H	-	+	-	+	-
73-9056	0149:H19	H	-	+	-	+	-
73-3297	0149:H19	H	-	+	+	+	+
75-750Lg	0149:H19	H	-	+	+	+	+
75-820	0149:H19	H	-	+	+	+	+
73-1929	0149:H19	H	-	+	+	+	-
74-3256	0149:H19	NH	-	+	+	+	-
73-3752	0149:NM	H	+	+	+	+	-

[a] Hemolysis on blood agar (5% sheep blood)
[b] H, hemolytic
[c] NT, not tested
[d] NH, nonhemolytic

Table 2. Heat- and antiserum-inactivation of LT produced by bovine strains of *E. coli*

		Heat Inactivation		Antiserum Inactivation	
Isolate	Serotype	65 C/30 min[a]	85 C/30 min[a]	Cell Culture[a]	Calf Loop
75-750Lg	0149:H19	-[b]	-	-	+
75-820	0149:H19	±	-	-	+
75-1611	0149:H19	-	-	-	-
73-1929	0149:H19	-	-	-	+
73-3738	0149:H19	±	-	-	-
73-3752	0149:NM	-	-	-	-
73-9056	0149:H19	-	-	-	-
74-3256	0149:H19	-	-	-	-

[a] Assayed in Y1 adrenal cell culture

[b] Negative (-) indicates no toxic response in cell culture; positive (+) indicates toxic response in cell culture; equivocal responses are indicated by ±.

media to compare the amount of enterotoxin produced in each medium. The culture supernatants were titered in two-fold dilutions and tested in suckling mice for ST activity. The largest amount of ST was produced in tryptic soy broth and this medium was used for all further enterotoxin production (Table 3).

Bovine ST purification

The purification of the heat stable enterotoxin using the methanol extraction method resulted in very small quantities of toxin. Therefore, the ultrafiltration method was the method of choice to remove protein with molecular weight greater than 10,000. The suckling mouse test for ST assay showed a greater yield of toxin when the ultrafiltration method was used.

A sephadex G-25 column was used to further purify the reconstituted protein. The ST activity was found to be present in the second absorbance peak (fractions 67-84, Figure 7). This peak contained approximately 22 mg/ml of protein after it was lyophilized and reconstituted in 0.05 M phosphate buffer. A dose of 270 ug/mouse was found to be positive for ST. The crude enterotoxin preparation contained 7 mg/ml of protein and 87.5 ug provided a positive response in mice.

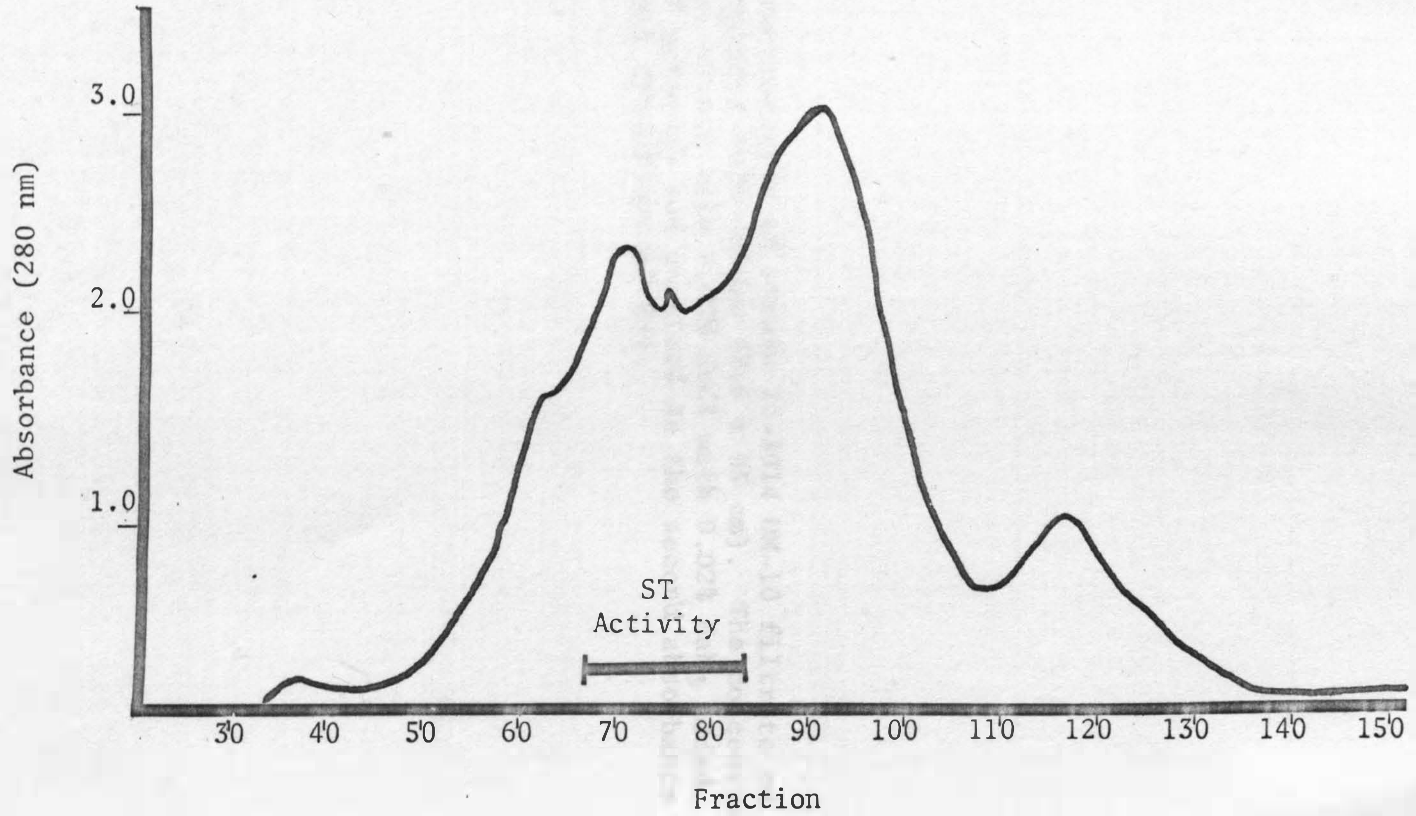
An ion exchange column of Sephadex A-50 prepared in 0.05 M phosphate buffer at pH 6.3 was found to be unsatisfactory for further purification because the toxin was not retained, but was eluted with 0.05 M phosphate buffer at pH 6.3. No toxin could be detected in the absorbance peak fractions eluted with 0.01 M, and 0.03 M NaCl in phosphate buffer. The ST active peak contained 2.5mg/ml of protein and 120 ug produced ST activity in mice (Figure 8).

Table 3. Comparison of three different media for *E. coli* production of ST assayed by the suckling mouse test

Isolate	Dilution	TSB[a]	Syncase[b]	Peptone Dialysate[b]
74-3717 (0138:NM)	1:1	0.1553[c]	0.1496	0.1471
	1:2	0.1367	0.1416	0.1429
	1:4	0.1233	0.1390	0.1308
	1:8	0.11945	0.1168	0.1119
	1:16	0.1093	0.0975	0.1102
74-656 (09:NM)	1:1	0.1354	0.1228	0.1371
	1:2	0.1427	0.1154	0.1071
	1:4	0.1226	0.0820	0.0878
	1:8	0.1061	0.0669	0.0667
	1:16	0.1034	0.0680	0.0631
74-5208 (020:NM)	1:1	0.1373	0.1149	0.1299
	1:2	0.1169	0.0856	0.0955
	1:4	0.1014	0.0863	0.0729
	1:8	0.0847	0.0618	0.0626
	1:16	0.0648	0.0579	0.0692

[a] Tryptic Soy Broth (Difco)
[b] For specific makeup of these media see Materials and Methods
[c] A gut to body weight ratio of $\geq$ 0.0900 was considered positive

Figure 7. Fractionation of strain 74-3914 UM-10 filtrate on a Sephadex G-25 column (2.5 x 95 cm). The concentrate was eluted with 0.02M NaCl with 0.02% NaN_3 added. ST activity was observed in the second absorbance peak (fractions 67-84).


Absorbance (280 nm)
3.0
2.0
1.0
ST
Activity
30
40
50
60
70
80
90
100
110
120
130
140
150
Fraction

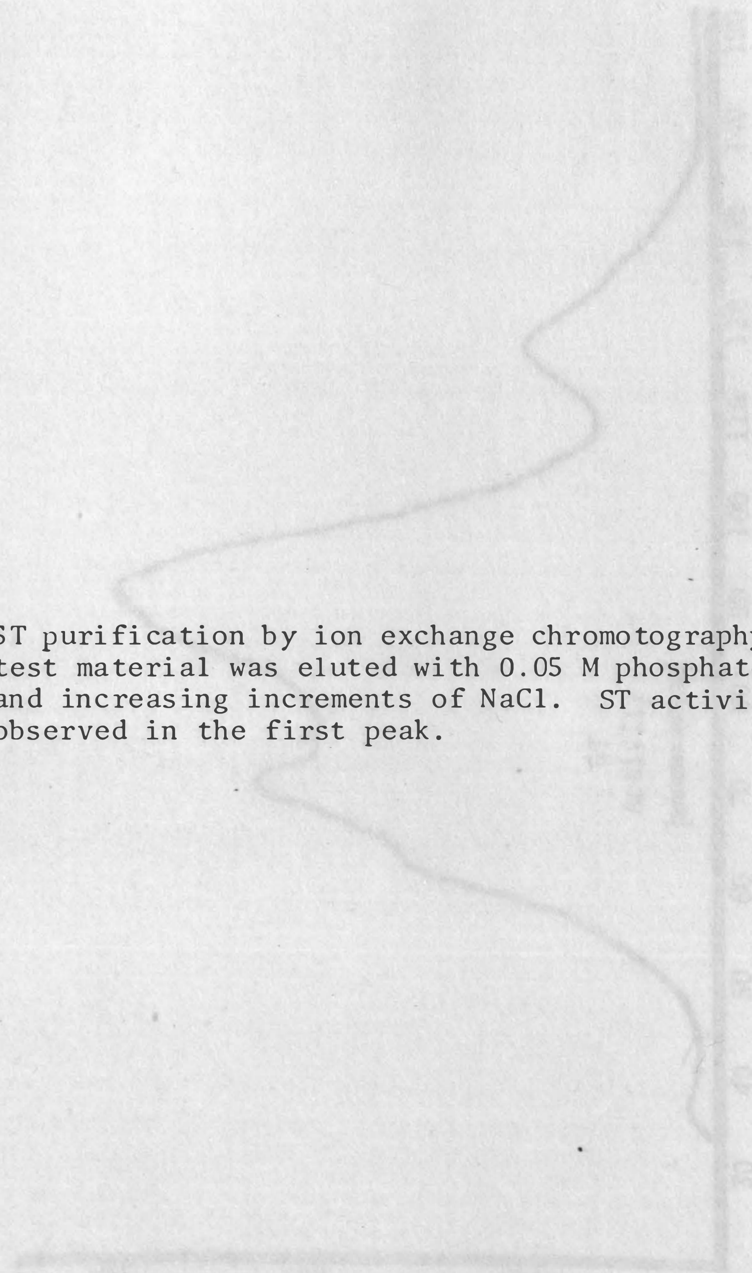
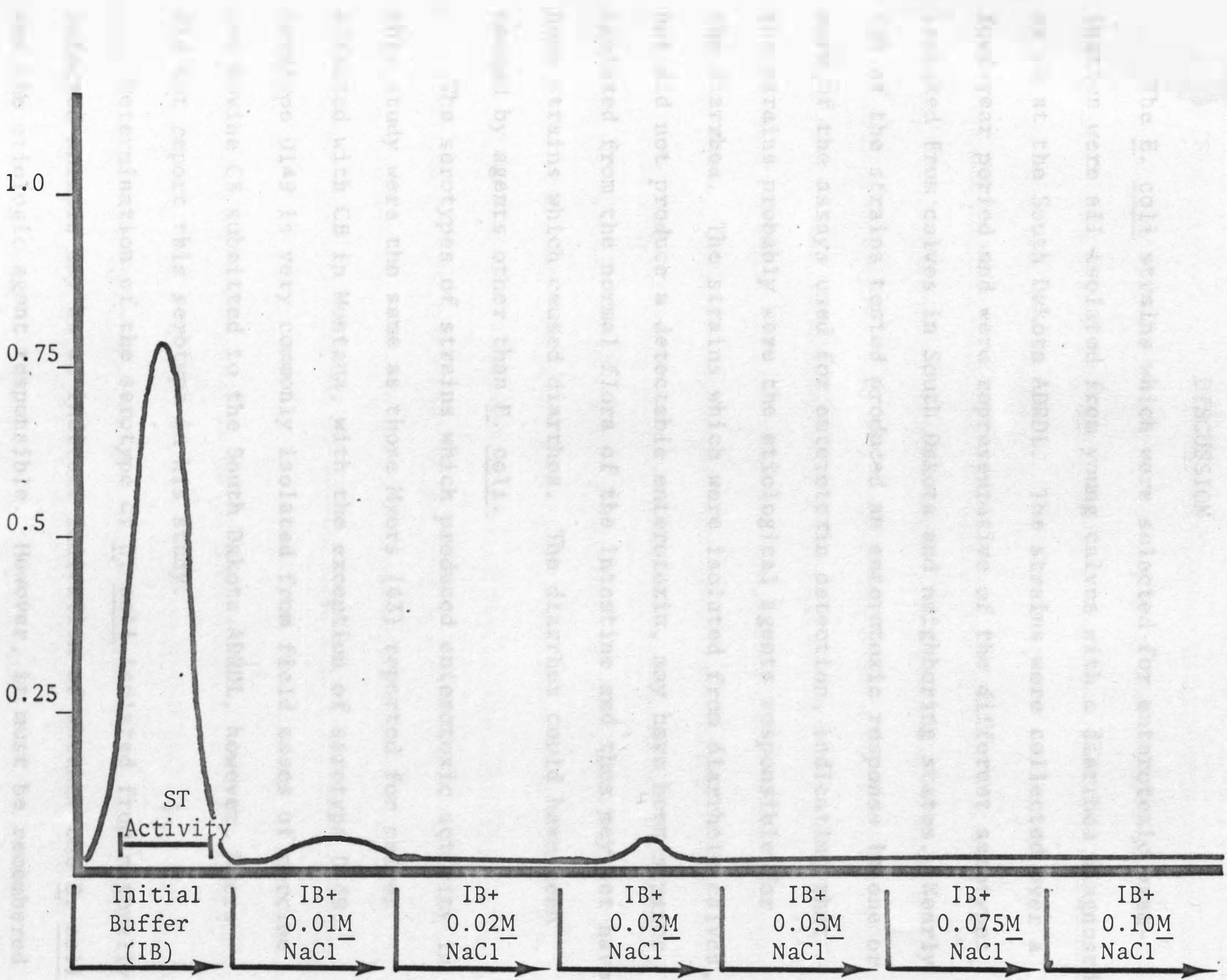


Figure 8. ST purification by ion exchange chromotography. The test material was eluted with 0.05 M phosphate buffer and increasing increments of NaCl. ST activity was observed in the first peak.


1.0
0.75
0.5
0.25
Absorbance (280 nm)
ST
Activity
Initial
Buffer
(IB)
IB+
0.01M
NaCl
IB+
0.02M
NaCl
IB+
0.03M
NaCl
IB+
0.05M
NaCl
IB+
0.075M
NaCl
IB+
0.10M
NaCl

DISCUSSION

The *E. coli* strains which were selected for enterotoxic examination were all isolated from young calves with a diarrhea diagnosed as CB at the South Dakota ADRDL. The strains were collected over a four-year period and were representative of the different serotypes isolated from calves in South Dakota and neighboring states. Nearly 50% of the strains tested produced an enterotoxic response in one or more of the assays used for enterotoxin detection, indicating that the strains probably were the etiological agents responsible for the diarrhea. The strains which were isolated from diarrheic calves, but did not produce a detectable enterotoxin, may have been strains isolated from the normal flora of the intestine and thus may not have been strains which caused diarrhea. The diarrhea could have been caused by agents other than *E. coli*.

The serotypes of strains which produced enterotoxic activity in this study were the same as those Myers (43) reported for calves affected with CB in Montana, with the exception of serotype 0149. Serotype 0149 is very commonly isolated from field cases of porcine and bovine CB submitted to the South Dakota ADRDL, however, Myers did not report this serotype in his study.

Determination of the serotype of *E. coli* isolated from naturally infected animals may be an indirect indication of whether the *E. coli* was the etiologic agent responsible. However, it must be remembered that enterotoxin production, the direct cause of the diarrhea, is the result of possession of a particular transmissible plasmid (21, 22, 51), and

is not due to somatic or capsular antigens. It is possible that the serotypes commonly associated with CB are more susceptible to conjugation, thus these serotypes possess the enterotoxin plasmid more frequently than other serotypes.

Since serotyping is not an accurate assessment of enterotoxigenicity, other diagnostic methods have been developed to detect enterotoxin production. The porcine LIS appeared sensitive to LT but not to ST. Bovine LIS were sensitive to both ST and LT, but there was a great variability between individual animals and between segments in one animal. There was very good correlation between the results of Y1 adrenal cell culture (sensitive only to LT) and porcine LIS, however the cell culture assay seems to be more sensitive than LIS to LT. The suckling mouse test (sensitive only to ST) detected ST in more enterotoxin than LIS of one of the calves, but this may have been due to the condition of the calf's intestine at the time of the LIS procedure. The other calf correlated exactly with the suckling mouse test, however the animal seemed to show several false positives.

The bovine LIS showed more variability between animals than did the porcine LIS. H.W. Moon (personal communcation) has also encountered this problem. The porcine LIS were not sensitive to ST, therefore it appears that LIS performed in one species are not reliable for the detection of ST and/or LT produced by all _E. coli_. The suckling mouse and Y1 adrenal cell culture assays are much quicker, less expensive, more consistent, and far less cumbersome than the LIS. From the

results obtained in this investigation, it appears that the suckling mouse and Y1 adrenal cell culture assays could be used in place of LIS.

Another possible explanation for the apparent discrepancies between detection of ST and LT in LIS or in suckling mouse and Y1 adrenal cell culture assays could be related to the amount of enterotoxin needed to produce a positive response in any of the tests. Bacteria-free supernatants were used in all investigations reported herein. Possibly more segments would have been positive if live organisms were used. The live organisms could attach to epithelial cells and the enterotoxins produced would be able to come into contact with the fluid secreting cells much faster and more often than when cell-free fluid is injected into the lumen of the intestine. Also the mucus and other secretions of the intestine could protect the cells from contact with the enterotoxin molecules. The environment of the small intestine may provide the optimum condition for enterotoxin production by _E. coli_ which has not been reproduced in synthetic media so that much more enterotoxin may be produced _in vivo_ than _in vitro_.

The discovery that eight of the bovine _E. coli_ strains produced LT is very interesting. LT produced by _E. coli_ isolated from ruminants has not been reported. Moon (38) stated that only ST has been found in ruminants. The LT activity from the eight strains was completely inactivated at 85 C and was neutralized with antiserum produced against porcine LT. These factors indicate the LT produced by bovine _E. coli_ is the same as the LT produced by porcine strains. That all LT produced by _E. coli_ strains are the same could be a very important factor in producing a vaccine which would produce LT antisera.

Smith and Gyles (49) reported that all enterotoxigenic strains of *E. coli* produced ST or ST and LT, but never LT alone. The eight LT producing strains could not be shown, by the assay methods used in the investigation, to produce ST. It is possible that these strains do produce ST, but in amounts too small to be detected by the suckling mouse and LIS assays.

Several media have been used for production of ST and LT by *E. coli* (2, 8, 10, 14, 31, 35, 42, 51). It was decided to compare TSB, syncase, and peptone dialysate to determine if *E. coli* would produce significantly higher amounts of ST in one medium than in the other two. Production of ST, when assayed in suckling mice, was the highest when the *E. coli* were grown in TSB. Therefore, TSB was the medium used for all ST production for purification efforts.

Prefiltering the medium with UM-50, UM-10, and UM-2 UF membranes was attempted so that the molecular weights of all growth materials in the medium would be smaller than ST (1,000 MW). Utilizing a medium prepared in this manner, the medium could then be refiltered, following enterotoxin production, to remove the remaining medium and the retentate would contain metabolic by-products, including the ST. No ST was produced using the prefiltered medium indicating that some essential growth factors for the production of ST were removed in the filtering process. Possibly a chemically defined medium with all constituents less than 1,000 MW could be developed which would still allow ST production.

Tryptic soy broth medium provided satisfactory results for the investigations reported herein. Sufficient amounts of ST were produced for purification efforts and it did not interfere with enterotoxin assays or neutralization tests.

The ultrafiltration method (24) which selectively removes the high-molecular weight components from the supernatant was found to yield greater quantities of ST than the methanol extraction method (33). The filtrate from either method contained several proteins as indicated by UV absorbance peaks observed when the concentrates were eluted from the Sephadex G-25 column. The ST activity was detected in the second absorbance peak which was collected in fractions 67-84. Possibly a longer column would have increased the resolution of the peaks. Ultrafiltration and G-25 chromatography results indicated that the MW of ST is between 1,000 and 10,000, which agrees with a previous report by Jacks (24).

ST activity was eluted in the first absorbance peak from the Sephadex A-50 ion exchange chromatography column. Elution of ST in the first peak indicated that the ST molecule did not adsorb onto the ion exchange beads. The experiment should be revised so that the pH at which the ST would remain on the column could be determined and then different ionic strengths of NaCl could be used to find the ionic strength at which the ST would be displaced. Ion exchange chromatography could be very valuable in obtaining large amounts of ST for further purification.

The original supernatant contained the greatest amount of ST activity. The first peak material from the ion exchange contained the next highest specific activity. The largest amount of activity was lost between UF and the gel filtration chromotography. A small amount of activity was lost on the Sephadex G-25 columns. Ion exchange chromatography increased ST activity relative to previous purification steps, however, the activity still did not reach the level of the original supernatant. The ST molecule may be unstable accounting for some of the decrease in activity during purification procedures. Washing the retentate of each of the ultrafiltration membranes several times might recover some of the ST lost in ultrafiltration process.

SUMMARY AND CONCLUSIONS

The enterotoxin production of 37 *E. coli* strains isolated from naturally occurring cases of calf colibacillosis was assayed in suckling mice, Y1 adrenal cell culture, porcine ligated intestinal segments (LIS), and bovine LIS. Nearly 50% of the strains produced heat-labile enterotoxin (LT), heat-stable enterotoxin (ST) or both. The results indicate that the suckling mouse and Y1 adrenal cell culture assays could be used in place of LIS for detection of enterotoxin.

The LT activity produced by bovine strains was completely neutralized at 85 C/30 min and anti-porcine LT sera completely neutralized bovine LT activity indicating that porcine LT and bovine LT are probably the same.

Three media, tryptic soy broth (TSB), syncase, and peptone dialysate, were compared for ST production. The largest amount of ST was produced in TSB.

Ultrafiltration, gel filtration chromatography, and ion exchange chromatography were used in ST purification. ST activity was obtained in the UM-10 filtrate, in the second absorbance peak on Sephadex G-25, and in the first absorbance peak on a Sephadex A-50 ion exchange gel. The ultrafiltration and gel filtration chromatography methods were suitable for purification of ST. However, the ion exchange chromatography proved unsatisfactory for concentration and purification of ST.

LITERATURE CITED

1. Barnum, D.A., P.J. Glantz, and H.W. Moon. 1965. Colibacillosis. CIBA Pharmaceutical Company. Summit, New Jersey.

2. Bywater, R.J. 1972. Dialysis and ultrafiltration of a heat-stable enterotoxin from Escherichia coli. J. Med. Microbiol. 5:337-343.

3. Craven, J.A., O.P. Miniats, and D.A. Barnum. 1971. Role of colicins in antagonism between strains of Escherichia coli in dual-infected gnotobiotic pigs. Am. J. Vet. Res. 32:1775-1779.

4. De, S.N., K. Bhattacharza, and J.K. Sarkar. 1956. A study of the pathogenicity of strains of Bacterium coli from acute and chronic enteritis. J. Path. Bact. 71:201-209.

5. De, S.N., M.L. Ghose, and A. Sen. 1960. Activities of bacteria-free preparations from Vibrio cholerae. J. Path. Bact. 79:373-380.

6. Dean, A.G., Y-C. Ching, R.G. Williams, and L.B. Harden. 1972. Test for Escherichia coli enterotoxin using infant mice: application in a study of diarrhea in children in Honolulu. J. Inf. Dis. 125:407-411.

7. Donta, S.T., H.W. Moon, and S.C. Whipp. 1974. Detection of heat-labile Echerichia coli enterotoxin with the use of adrenal cells in tissue culture. Sci. 183:334-336.

8. Dorner, F. 1975. Escherichia coli enterotoxin: purification and partial characterization. J. Biol. Chem. 250:8712-8719.

9. Dorner, F., and P. Mayer. 1975. Escherichia coli enterotoxin: stimulation of adenylate cyclase in broken cell preparations. Inf. Immun. 11:429-435.

10. Ellis, R.P., R.L. Pierce, C.A. Kirkbride, and M.M. Kieffer. 1973. Use of suckling mice for detection of enteropathogenicity of Escherichia coli isolated from calves and pigs. Proceedings 77th An. Meeting US Animal Health Ass. p. 644-649.

11. Evans, D.G., D.J. Evans, and N.F. Pierce. 1973. Differences in the response of rabbit small intestine to heat-labile and heat-stable enterotoxins of Escherichia coli. Inf. Immun. 7:873-880.

12. Evans, D.J., D.G. Evans, and S.L. Gorbach. 1974. Polymyxin B-induced release of low-molecular-weight, heat-labile enterotoxin from Escherichia coli. Inf. Immun. 10:1010-1017.

13. Ferguson, W.W., and R.C. June. 1952. Experiments on feeding adult volunteers with Escherichia coli III B_4, a coliform organism associated with infant diarrhea. Amer. J. Hyg. 55:155-161.

14. Finkelstein, R.A., P.A. Sobocinski, P. Attbasampanna, and P. Charunmethee. 1966. Pathogenesis of experimental cholera: identification of choleragen (procholeragen A) by disc immunoelectrophoresis and its differentiation from cholera mucinase. J. Immunol. 97:25-33.

15. Finkelstein, R.A., M.L. Vasil, J.R. Jones, R.A. Anderson, and T. Barnard. 1976. Clinical cholera caused by enterotoxigenic Escherichia coli. J. Clin. Microbiol. 3:382-384.

16. Gay, C.C. 1965. Escherichia coli and neonatal disease of calves. Bacteriol. Rev. 29:75-101.

17. Glantz, P.J. 1971. Serotypes of Escherichia coli associated with colibacillosis in neonatal animals. Ann. N.Y. Acad. Sci. 176:67-79.

18. Goldschmidt, R. 1933. Untersuchungen zur atiology der durch falserkrankungen des sauglings. Jahrb. Kinderheilk. 89:318-324.

19. Guerrant, R.L., L.L. Brunton, T.C. Schnaitman, L.I. Rebhun, and A.G. Gilman. 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for the enterotoxins of Vibrio cholerae and Escherichia coli. Inf. Immun. 10:320-327.

20. Gyles, C.L. 1971. Heat-labile and heat-stable forms of enterotoxin from E. coli strains enterophathogenic for pigs. Ann. N.Y. Acad. Sci. 1976:314-322.

21. Gyles, C.L., and D.A. Barnum. 1969. A heat-labile enterotoxin from strains of Escherichia coli enteropathogenic for pigs. J. Inf. Dis. 120:419-426.

22. Gyles, C.L., M. So, and S. Falkow. 1974. The enterotoxin plasmids of Escherichia coli. J. Infect. Dis. 130:40-49.

23. Isaacson, R.E., and H.W. Moon. 1975. Induction of heat-labile enterotoxin synthesis in enterotoxigenic Escherichia coli by Mitomycin C. Inf. Immun. 12:1271-1275.

24. Jacks, T.M., and B.J. Wu. 1974. Biochemical Properties of Escherichia coli low-molecular-weight, heat-stable enterotoxin. Inf. Immun. 9:342-347.

25. Jacks, T.M., B.J. Wu, A.C. Braemer, and D.E. Bidlack. 1973. Properties of the enterotoxic component in Escherichia coli enteropathogenic for swine. Inf. Immun. 7:178-189.

26. Jensen, C.O. 1913. Handbuch der pathogen microorganismen. Kolle and Wasserman. 6:131-138.

27. Kantor, H.S. 1975. Enterotoxins of Escherichia coli and Vibrio cholerae: tools for the molecular biologist. J. Infect. Dis. 131:522-531.

28. Kantor, H.S., P. Tao, and S.L. Gorbach. 1974. Stimulation of intestinal adenyl cyclase by Escherichia coli enterotoxin: comparison of strains from an infant and an adult with diarrhea. J. Infect. Dis. 129:1-9.

29. Kauffmann, F. 1942. The serology of the coli group. J. Immunol. 57:71-77.

30. Keusch, G.T., and S.T. Donta. 1975. Classification of enterotoxins on the basis of activity in cell culture. J. Infect. Dis. 131:58-63.

31. Kohler, E.M. 1968. Enterotoxic activity of filtrates of Escherichia coli in young pigs. Am. J. Vet. Res. 29:2263-2274.

32. Kohler, E.M. 1971. Observation on enterotoxins produced by enteropathogenic Escherichia coli. Ann. N.Y. Acad. Sci. 176:212-219.

33. Kohler, E.M. and E.H. Bohl. 1966. Studies of Escherichia coli in gnotobiotic Pigs. I. Experimental reproduction of colibacillosis. Canad. J. Comp. Med. Sci. 30:199-203.

34. Kwan, C.N., and R.M. Wishnow. 1974. Escherichia coli enterotoxin-induced steroidogenesis in cultured adrenal tumor cells. Inf. Immun. 10:146-151.

35. Lariviere, S., C.L. Gyles, and D.A. Barnum. 1973. Preliminary characterization of the heat-labile enterotoxin of Escherichia coli F11(P155). J. Infect. Dis. 128:312-320.

36. Lovell, R. 1937. Classification of Bacterium coli from diseased calves. J. Path. Bact. 44:125-130.

37. Mitchell, I.G., M.J. Tame, and R. Kenworthy. 1974. Separation and purification of enterotoxins from a strain of Escherichia coli pathogenic for pigs. J. Med. Microbiol. 7:439-450.

38. Moon, H.W. 1974. Pathogenesis of enteric diseases caused by Escherichia coli, p. 179-211. In C.A. Bradley and C.E. Cornelius (ed.), Advances in Veterinary Science and Comparative Medicine. Academic Press Inc., New York and London.

39. Moon, H.W., N.O. Nielsen, and T.T. Kramer. 1970. Experimental enteric colibacillosis of the newborn pig: histopathology of the small intestine and change in plasma electrolytes. Am. J. Vet. Res. 31:103-112.

40. Moon, H.W., and S.C. Whipp. 1970. Development of resistance with age by swine intestine to effects of enteropathogenic Escherichia coli J. Inf. Dis. 122:220-223.

41. Moon, H.W., S.C. Whipp, and A.L. Baetz. 1971. Comparative effects of enterotoxins from Escherichia coli and Vibrio cholerae on rabbit and swine small intestine. Lab. Invest. 25:133-140.

42. Moon, H.W., S.C. Whipp, G.W. Engstom, and A.L. Baetz. 1970. Response of the rabbit ileal loop to cell-free products from Escherichia coli enteropathogenic for swine. J. Infect. Dis. 121:182-187.

43. Myers, L.L., and P.A.M. Guinee. 1976. Occurrence and characteristics of enterotoxigenic Escherichia coli isolated from calves with diarrhea. Inf. Immun. 13:1117-1119.

44. Nagy, B., H.W. Moon, and R.E. Isaacson. 1976. Colonization of porcine small intestine by Escherichia coli: ileal colonization and adhesion by pig enteropathogens that lack K88 antigen and by some acapsular mutants. Inf. Immun. 13:1214-1220.

45. Nalin, D.R., A.I. Bhattachorjee, and S.H. Richardson. 1974. Cholera-like toxic effect of culture filtrates of Escherichia coli. J. Infect. Dis. 130:595-601.

46. Newman, F.S., L.L. Myers, B.D. Firehammer, and J.E. Catlin. 1973. Prevention of experimentally induced enteric colibacillosis in newborn calves. Inf. Immun. 8:540-543.

47. Rutter, J.M., and P.D. Luther. 1973. Cytopathic factors in bacteria-free lysates of Escherichia coli. J. Med. Microbiol. 6:565-571.

48. Sack, D.A., and R.B. Sack. 1975. Test for enterotoxigenic Escherichia coli using Y1 adrenal cells in miniculture. Inf. Immun. 11:334-336.

49. Smith, H.W., and C.L. Gyles. 1970. The relationship between two apparently different enterotoxins produced by enteropathogenic strains of Escherichia coli of porcine origin. J. Med. Microbiol. 3:387-401.

50. Smith, H.W., and S. Halls. 1967. Observations by the ligated intestinal segment and oral innoculation methods on Escherichia coli infections in pigs, calves, lambs, and rabbits. J. Path. Bact. 93:499-529.

51. Smith, H.W., and S. Halls. 1967. Studies on Escherichia coli enterotoxin. J. Path. Bact. 93:531-543.

52. Smith, H.W., and S. Halls. 1968. The transmissible nature of the genetic factor in Escherichia coli that controls enterotoxin production. J. Gen. Microbiol. 52:319-334.

53. Smith, H.W., and E.T. Jones. 1963. Observations on the alimentary tract and its bacterial flora in healthy and diseased pigs. J. Path. Bact. 86:387-412.

54. Smith, H.W., and M.A. Linggood. 1971. Observations on the pathogenic properties of K88, Hly, and Ent plasmids of Escherichia coli with particular reference to porcine diarrhea. J. Med. Microbiol. 4:467-485.

55. Smith, H.W. and M.A. Linggood. 1972. Further observations on Escherichia coli enterotoxins with particular regard to those produced by atypical piglet strains and by calf and lamb strains: the transmissible nature of these enterotoxins and of a K antigen possessed by calf and lamb strains. J. Med. Microbiol. 5: 243-250.

56. Smith, N.W., and R.B. Sack. 1973. Immunologic cross-reactions of enterotoxins from Escherichia coli and Vibrio cholerae. J. Infect. Dis. 127:164-170.

57. Smith, T., and G. Bryant. 1927. Studies on pathogenic *Bact. coli* from bovine sources. 2. Mutations and their immunological significance. J. Exptl. Med. 46:133-141.

58. Smith, T., and M.L. Orcutt. 1925. The bacteriology of the intestinal tract of young calves with special reference to early diarrhea. J. Exptl. Med. 41:89-94.

59. South Dakota Agriculture. South Dakota Crop and Livestock Reporting Service. 312 South Minnesota Avenue, Sioux Falls, SD. 1974.

60. Stevens, H.J. 1963. Enteritis in Pigs. 1. Coliform infections in the young pig and a practical approach to the control of enteritis. Vet. Rec. 75:1241-1246.

61. Taylor, J., and K.A. Bettelheim. 1966. The action of chloroform-killed suspensions of enteropathogenic Escherichia on ligated rabbit-gut segments. J. Gen. Microbiol. 43:309-313.

62. Taylor, J., M.P. Maltby, and J.M. Payne. 1958. Factors influencing the response of ligated rabbit gut segments to injected *Escherichia coli*. J. Path. Bact. 76:491-499.

63. Truszcynski, M., and J. Pilaszek. Effects of injection of enterotoxin, endotoxin or live culture of *Escherichia coli* into small intestine of pigs. Res. Vet. Sci. 10:469-476.