INFECTION AND IMMUNITY, Feb. 1976, p. 373-381

Vol. 13, No. 2
Printed in U.S.A.

Purification and Properties of *Klebsiella pneumoniae* Heat-Stable Enterotoxin

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Received for publication 31 July 1975

The enterotoxic material in cell-free growth preparations of *Klebsiella pneumoniae* serotype 5 was purified by sequential ultrafiltration and gel filtration (GF) procedures and the fractions were assayed for enterotoxic activity by determining their ability to induce in vivo net water secretion in the rat jejunum. Whole-cell lysates were inactive. Anaerobic broth culture conditions yielded a 10-fold increase in toxin production over aerobic conditions. Enterotoxic activity was absent in the UM-10 retentate of the broth filtrate but present in both the retentate and filtrate of the UM-2 membrane. GF of the two UM-2 ultrafiltration fractions through a Sephadex G-25 column yielded an active eluate, whose potency was increased by 10- or 200-fold, in or adjacent to the void volume. When subsequently passed through a G-50 column, these pools eluted at a K_{av} of between 0.4 and 0.6 and were further increased in potency by two- or fivefold. A second equally potent fraction was also recovered in the void volume of the G-50 eluate of the UM-2 filtrate; this may represent a polymer. Progressive purification by GF was associated with an increased protein and decreased carbohydrate content of the most active fractions. The most active G-50 eluate of the UM-2 retentate had a minimal effective enterotoxic dose of 5 μg/ml and that of the filtrate was less than 0.1 μg/ml. Heating the active GF eluates to 100 C for 30 min did not abolish enterotoxic activity and lowering the pH to 1 or incubation with either Pronase or trypsin had no effect on activity. These observations indicate that *K. pneumoniae* heat-stable enterotoxin is probably a single toxin with an apparent molecular weight in the range of 5,000. The elution characteristics during GF as well as the chemical composition of the most purified enterotoxin fractions indicate that the toxin is not associated with endotoxin.

It is now well established that transient colonization of the small intestine by noninvasive enterotoxigenic strains of *Escherichia coli* is a common cause of acute diarrheal episodes among both visitors to and the indigenous population of tropical areas (4, 10, 11, 35). Strains of *Klebsiella pneumoniae* are also commonly isolated in the tropics from the small bowel of persons with acute diarrhea (3, 10), malnourished children who have chronic diarrhea (12, 15), and individuals with overt tropical sprue (20, 24). Although recognized to be associated with acute diarrhea in children (32, 39), klebsiella has not previously been considered pathogenic when resident within the intestinal tract. Recently, however, strains of *K. pneumoniae* isolated from the jejunum of both Puerto Ricans (20) and Haitians (24) with tropical sprue have been shown to elaborate an enterotoxin that induces net secretion of water and electrolytes, impaired absorption of xylose, and structural abnormalities of the intestinal mucosa in several different experimental animal models (21, 22).

We have recently described some of the characteristics of the enterotoxin elaborated by a strain of *K. pneumoniae* isolated from a Puerto Rican with tropical sprue (23). The toxin preparation is heat and acid stable and dialyzable through viscose tubing. After sequential passage of an acetone precipitate of the broth filtrate through various-sized ultrafiltration (UF) membranes, toxin activity is present only in those fractions with a molecular weight of less than 10,000. In the present study, we have evaluated the optimum culture conditions for production of this toxin, applied additional purification procedures using sequential gel filtration chromatography to the UF fractions, and char-

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acterized some of the properties of the purified material thus obtained. Enterotoxigenic activity of the fractions was ascertained by direct quantitative determination of their ability to induce net secretion of water in the rat jejunum using standard in vivo marker perfusion techniques.

MATERIALS AND METHODS

Organism. A strain of *K. pneumoniae* serotype 5, API biotype no. 5215773 (37), which was the predominant organism cultured from the midjejunal aspirate of a Puerto Rican with tropical sprue (20) and which we have examined in previous studies (21–23), was used.

Enterotoxin production. Whole-cell lysates were prepared by sonication (5) of cells harvested after 18-h aerobic culture on Trypticase soy agar (TSA) (Baltimore Biological Laboratory, Cockeysville, Md.). Broth cultures were prepared by 18-h aerobic stationary or agitated incubation in 250 ml of either Trypticase soy broth (TSB) or syncase broth (8), with glucose substituted for sucrose, in a 2-liter flask. For anaerobic cultures, bacteria were inoculated into 500 ml of broth in a 1-liter flask and incubated for 18 h in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.). The broth cultures and sonically treated material were centrifuged at 35,000 × *g* for 45 min and the supernatant was filtered through a 0.45-μm membrane filter (Millipore Corp.) adapted with a type AP 25 microfiber glass prefilter (Millipore Corp.). Material in the cell-free broth filtrates was then precipitated by the addition of 8 volumes of acetone at 4 C and further processed in the manner described previously by this (23) and other laboratories (2).

UF. The acetone precipitates were all subjected to sequential at UF at 4 C through Amicon UM-10 and UM-2 membranes (Amicon Corp., Lexington, Mass.). Unless otherwise specified, the retentates of these membranes were taken to 10% of the original volume without washing and they and the UM-2 filtrate were then concentrated by lyophilization and stored at −20 C until assay or further purification procedures.

Gel filtration chromatography. A column (5 by 85 cm) was packed with Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N.J.) and equilibrated with 0.02 M ammonium bicarbonate (pH 7.8). Excluded and included volumes were determined using blue dextran 2000 (Pharmacia) and NaCl. A 200- to 500-mg amount of either the UM-2 retentate or filtrate, dissolved in 10 ml of the above buffer, was eluted from the gel at 4 C at a flow rate of 24 ml/h. The eluate was collected in 20-ml fractions; each tube was monitored at 280 nm for protein content and every third tube at 620 nm for total hexose content, as determined by the anthrone reaction (38), on a model 139 Hitachi Perkin-Elmer spectrophotometer. The fractions were then separated into pools which were concentrated by lyophilization. From 20 to 31 mg of the most active G-25 pool, derived from combining the pools from three previous gel separations, was then placed on a Sephadex G-50 column of the same dimensions and eluted under the identical conditions as the G-25 column.

Chemical analysis. Protein concentrations were determined by the method of Lowry et al. (28), using bovine serum albumin as the standard, and carbohydrate was determined by the anthrone reaction (38), using mannose as the standard. Polyethylene glycol was determined by a modification (29) of Hyden's turbidimetric technique (17). Osmolalities were determined by measuring the freezing point depression with an Advanced Digimatic Osmometer.

Effect of proteolytic enzymes. One-milligram quantities of the most active GF elution pools of the UM-2 retentate and filtrate were mixed with Pronase (Calbiochem, LaJolla, Calif.) in a substrate-to-enzyme ratio, based on protein concentration, of 10:1 and incubated in 0.01 M phosphate buffer, pH 7.4, at 37 C for 24 h (19). The same quantities of these fractions were also mixed in the same substrate-enzyme ratio with trypsin (Worthington Biochemicals, Freehold, N.J.) using tris(hydroxymethyl)aminomethane-hydrochloride buffer with a final calcium concentration of 0.001 M and a pH of 8.1, and then incubated at 37 for 3 h (18). Tryptic activity was confirmed with TAME (16). After incubation, the test fractions were perfused at a concentration of 10 μg/ml; in addition, both the Pronase and trypsin were perfused alone, in the same amount that was mixed with the toxin fractions, in other rats to serve as controls.

Assay for enterotoxic activity. Test fractions were assayed for their ability to induce net secretion of water in the rat jejunum by means of standard marker perfusion techniques for the in vivo evaluation of intestinal transport (22, 34). Single 20-cm jejunal segments were cannulated in 150-g Spraque-Dawley rats and perfused at a rate of 0.5 ml/min, using a model 1201 Harvard peristaltic pump. The test preparation was perfused during both a 30-min steady-state period and four 30-min test periods. The electrolyte solution described by Powell and Malawer (34), as modified in this laboratory (22) when necessary to maintain an iso-osmolality of 317 mOsm/kg, was used. Net transport of water was calculated by changes in the polyethylene glycol concentrations by the usual water marker technique equation (34). Values are expressed in microliters per centimeter per 30 min. Net lumen to blood transport is termed absorption, which is signified by a plus sign, whereas a minus sign refers to net blood to lumen transport, or secretion. We have previously reported (22), and so noted in the present study, that marginally active dosages of enterotoxin will induce net secretion during only one or two of the test periods. For this reason, the values reported are for that single 30-min test period during which there was minimal absorption or maximal secretion of water.

The minimal effective does (MED) of toxin is defined as that amount (in dry weight) of toxin that induces net secretion. Control preparations consisted of the cell-free sonic extract and the UM-2 retentate of an acetone precipitate after aerobic growth in TSB of a nontoxigenic strain of *E. coli* (API biotype no. 5044572) isolated from a control

TABLE 1. *Effect of modifications of growth procedures on enterotoxic activity*

Medium[a]	Method[b]	Water transport		
		2,000[c]	1,000	500
Aerobic				
TSA	Sonic extract	+34	+48	
TSB	Stationary	−43	−26	+46
TSB	Agitated	−36	+41	
SYN	Stationary	−13	+39	
SYN	Agitated	−2	+16	
Anaerobic				
TSB	Stationary		−163	−29

[a] All broth fractions are UM-2 retentates of the acetone precipitate. Syn, syncase broth.

[b] Agitated culture broths were shaken 200 times/min during growth.

[c] Dosage in micrograms per milliliter.

TABLE 2. *Enterotoxigenic activity of UF of the acetone precipitate after anaerobic growth*[a]

Membrane	Fraction	Washed	Water transport	
			100[a]	10
UM-10	Retentate	No	+50	
UM-2	Retentate	No	−12	+26
UM-2	Retentate	Yes	+2	
UM-2	Filtrate	No	−29	+33
UM-2	Filtrate	Yes	−31	+18

[a] Dosage in micrograms per milliliter.

Puerto Rican (20). When tested in groups of five rats each at a dosage of 2,000 μg/ml, these preparations gave minimal values of water absorption of +34 ± 4 (mean ± standard error of the mean) for the sonic extract and +36 ± 7 for the broth ultrafiltrate.

RESULTS

Influence of growth procedures on enterotoxin production. Since previous studies, which used the rabbit ileal loop as an assay model, demonstrated that toxigenic activity is absent from the UM-10 retentate and present principally in the UM-2 retentate of an acetone precipitate of the cell-free broth filtrates of the *K. pneumoniae* strain under study (23), the latter UF fraction was initially used to test for activity in preparations derived from using various broth culture materials and conditions (Table 1). The test fractions were initially screened for toxigenic activity at a dosage of 2,000 μg/ml and subsequently, if found active at this concentration, tested at lower dosages.

The whole-cell lysate derived from cell-free sonically treated material grown aerobically on TSA was inactive even at the highest dosage. Among the broth cultures grown under stationary aerobic conditions, the broth filtrate after growth in TSB was more active, with an MED of 1,000 μg/ml, than that grown in syncase broth. Using either of these two culture media, the material derived from agitated growth conditions, during which the culture was shaken 200 times per min, was less active than that obtained from the stationary cultures. Stationary growth in TSB under anaerobic conditions yielded the most potent material, with an MED of 100 μg/ml, and the activity of various UF fractions of material produced in this manner was next analyzed.

Activity of UF fractions. When tested at a concentration of 100 μg/ml, the UM-10 retentate was inactive (Table 2). Both the UM-2 retentate and filtrate were active, the latter being the more potent of the two. Washing the UM-2 retentate five times with 0.02 M ammonium bicarbonate, pH 7.8, reduced the activity in the retentate but did not change that of the filtrate. None of the UF fractions were active at a concentration of 10 μg/ml.

Fractionation by GF. (i) UM-2 retentate. The eluate from the Sephadex G-25 column (Fig. 1) was divided into four pools, each of which was initially screened for enterotoxigenic activity at a dosage of 100 μg/ml (Table 3). Pool 1, which consisted principally of the void volume, had several protein peaks and was the most active, with an MED of 10 μg/ml. Pool 2, which contained the first part of a second large protein peak, was less active, and both pool 3, which contained the peak carbohydrate activity, and pool 4 were inactive.

Three distinct areas of protein peaks were evident in the eluate after passage of the G-25 pool 1 through a G-50 column; in addition, several carbohydrate peaks were detected in subsequent elution fractions (Fig. 2). Each of the three areas of protein peaks was separated into a pool which was tested for activity at a dosage of 10 μg/ml (Table 3). The first two pools were inactive; the third, which had a K_{av} of between 0.41 and 0.58, had an MED of 5 μg/ml. A fourth pool, containing the carbohydrate peaks, was inactive.

(ii) UM-2 Filtrate. The eluate from the G-25 column was divided into four pools, two of which contained several large protein peaks (Fig. 3). These were tested at an initial dosage of 10 μg/ml. Pool 2, which had a K_{av} of between 0.14 and 0.28, was extremely active with an MED of 0.5 μg/ml. Both pool 3 and pool 4, which contained several protein and carbohydrate peaks, were inactive.

Four separate areas of protein peaks, but no

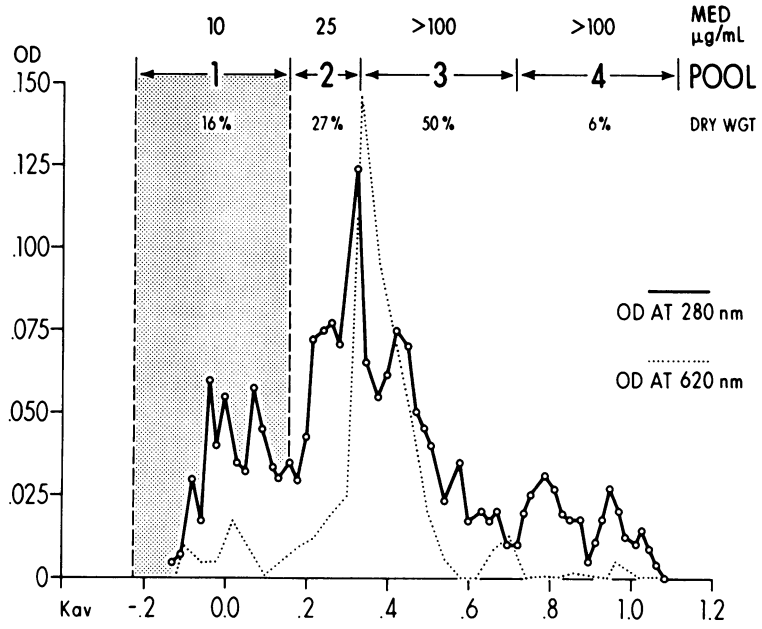

FIG. 1. *Gel filtration through a Sephadex G-25 column of the UM-2 retentate. Optical density values indicate protein content at 280 nm and carbohydrate content of 620 nm. The strippled area indicates the most active pool.*

TABLE 3. *Enterotoxic activity of gel filtration fractions of the UM-2 retentate*

Gel[a]	Pool	Water transport				
		100[b]	25	10	5	1
G-25	1	−45	−24	−3	+28	
G-25	2	−29	−6	+16		
G-25	3	+4				
G-25	4	+9				
G-50	1			+16		
G-50	2			+4		
G-50	3			−48	−36	+14
G-50	4			+16		

[a] G-25 GF of the UM-2 retentate. G-50 GF of pool 1 eluates from the G-25 separation.

[b] Dosage in micrograms per milliliter.

carbohydrate peaks, were evident when the G-25 pool 2 eluate was passed through a G-50 column (Fig. 4); these were each separated into a pool and tested at an initial concentration of 0.5 μg/ml (Table 4). Both pool 1, the void volume, and pool 3, which had a K_{av} of between 0.42 and 0.53, were active with an MED of <0.1 μg/ml; pools 2 and 4 were inactive.

Composition of the active fractions. Progressive purification of the UF fractions by GF was associated with an increased protein and a decreased carbohydrate content of the active material (Table 5). Purification of the UM-2 retentate by sequential GF resulted in a 20-fold increase in enterotoxic activity that was contained in a fraction consisting of 6.2% (approximately 1/16) of the original dry weight of the retentate. Similar treatment of the UM-2 filtrate produced a 1,000-fold increase in activity that was contained in two pools which consisted of approximately 0.14% (1/700) of the original dry weight of the filtrate.

Effect of physical changes or treatment with proteolytic enzymes. The effect of various physical treatments on the active GF eluates is shown in Table 6. There was an insufficient quantity of the G-50 eluate of the UM-2 filtrate, and therefore the G-25 eluate of this material was examined. Heat treatment, 100 C for 30 min, had no effect on the activity of the retentate G-50 eluate but it did reduce, but not abolish, the activity of the G-25 eluate of the filtrate. Acid treatment, consisting of lowering the pH to 1 for 4 h by the addition of concentrated HCl, and incubation with either Pronase for 24 h or trypsin for 3 h, did not reduce the enterotoxic activity of the purified fractions. Perfusion of a rat with 100 ml containing 32.7 μg of trypsin, the amount incubated with the toxin fractions, did not effect water transport; minimal absorption was +53. During perfusion with 33.8 μg of Pronase alone, water absorption was normal during the first two collection periods but fell to values of +2 and +11 during the

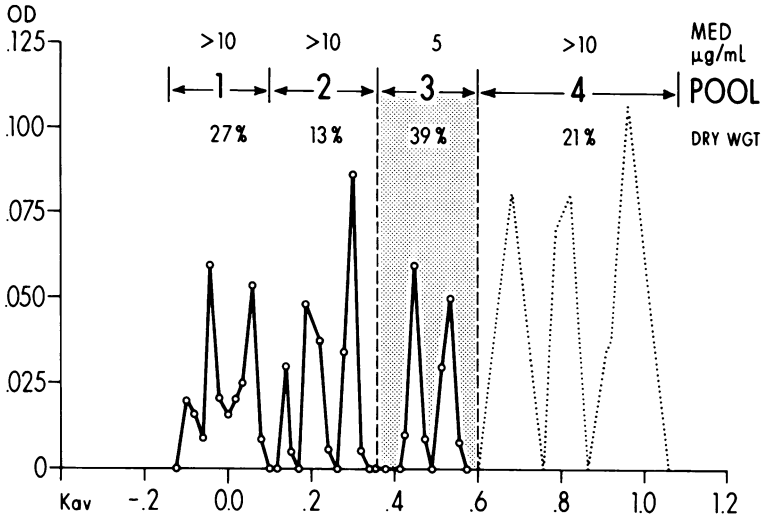

FIG. 2. *Gel filtration through a Sephadex G-50 column of pool 1 eluated from the G-25 GF of the UM-2 retentate.*

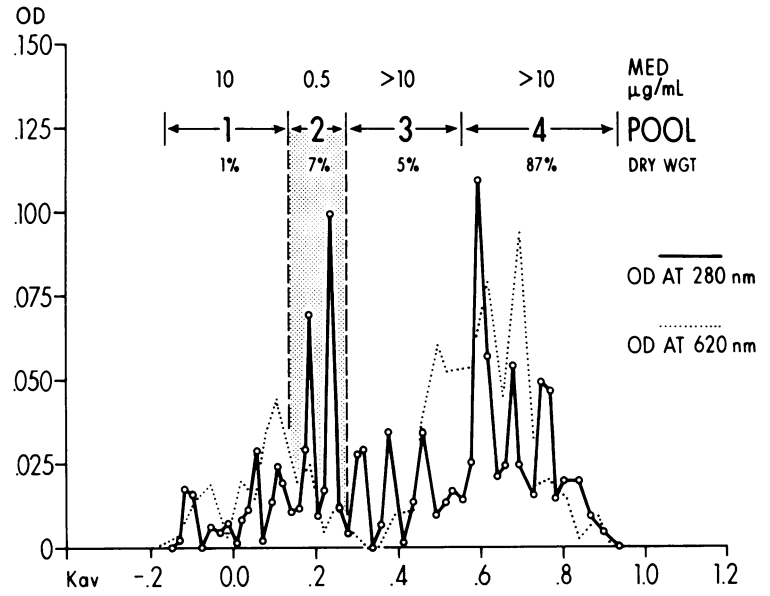

FIG. 3. *Gel filtration through a Sephadex G-25 column of the UM-2 filtrate.*

third and fourth collection periods. Since the peak secretory effect of the incubation mixtures of toxin plus Pronase occurred during the first collection period, we feel that this is evidence that Pronase fails to alter the enterotoxic activities of the test fractions rather than a manifestation of the effect of Pronase itself.

DISCUSSION

The results of the present studies indicate that the strain of *K. pneumoniae* under study produces a low-molecular-weight, heat-stable enterotoxin (ST) only. No activity was detected in cell-free whole cell lysates of bacterial growth, the standard procedure used to produce heat-labile toxin (LT) (5, 14, 36). Optimum conditions for *K. pneumoniae* ST production appear to be complete anaerobiasis. Material growth in this manner was 10 times more active than that after aerobic stationary growth which, in turn, was more active than that following the more aerobic condition associated

with agitation during culture. This observation may be of clinical significance since relatively anaerobic conditions prevail in the small intestine (27), the site of chronic contamination by *K. pneumoniae* in tropical sprue (20, 24), so that enhanced ST production might be anticipated in this environment. The conditions uniformly used by other laboratories for producing *E. coli* enterotoxins are aerobic growth, either stationary or agitated (6, 14, 18, 19, 25, 26, 30, 36). We are unaware of any published information concerning the effect of anaerobic culture conditions on *E. coli* enterotoxin production; in our laboratory, we find that anaerobic growth yields *E. coli* ST only (C. S. Lee, and F. A. Klipstein, unpublished observations).

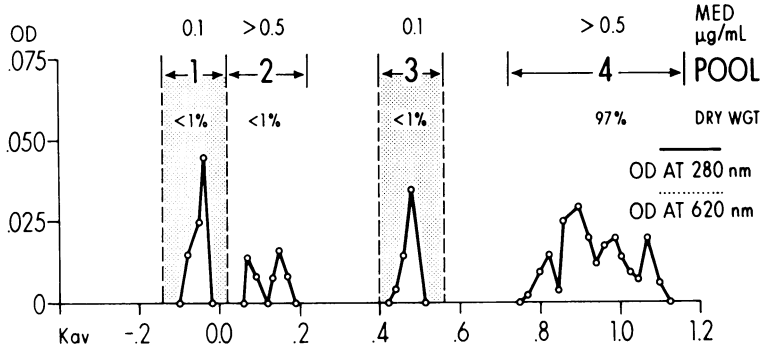

FIG. 4. *Gel filtration through a Sephadex G-50 column of pool 2 eluted from the G-25 GF of the UM-2 filtrate.*

TABLE 4. *Enterotoxic activity of gel filtration fractions of the UM-2 filtrate*

Gel[a]	Pool	Water transport			
		10[b]	1	0.5	0.1
G-25	1	−23	+5		
G-25	2	−57	−17	−5	+51
G-25	3	+33			
G-25	4	+8		−109	
G-50	1			+32	−6
G-50	2			−73	
G-50	3			+27	−46
G-50	4				

[a] G-25 GF of the UM-2 filtrate. G-50 GF of pool 2 eluates from the G-25 separation.
[b] Dosages in micrograms per milliliter.

In preliminary studies of this *K. pneumoniae* strain, in which we used the ligated rabbit ileal loop model as a test assay system (23), we found that after sequential ultrafiltration of an acetone precipitate of the broth filtrate through Amicon UM-10 and UM-2 membranes, enterotoxin activity was absent in the fraction containing material with a molecular weight of greater than 10,000 that was retained by the UM-10 membrane and present both in the UM-2 retentate and, to a lesser degree, the UM-2 filtrate fractions. Our observations in the present study, in which we have used a much more sensitive and quantitative biological assay system, are the same with the exception that the UM-2 filtrate appears to be the most active

TABLE 5. *Composition and enterotoxic activities of the most active fractions obtained during sequential purification procedures*

Fraction	Carbohydrate (%)	Protein (%)	MED[a] (dry wt) (μg/ml)	MED[a] protein (μg/ml)	Initial[b] wt (%)[b]
UM-2 retentate					
Retentate	11.2	25.2	100	25.2	28.2
G-25 pool 1	14.3	33.4	10	3.3	4.5
G-50 pool 3	2.8	43.4	5	3.1	1.9
UM-2 filtrate					
Filtrate	6.7	7.7	100	7.7	8.1
G-25 pool 2	1.2	31.3	0.5	0.16	0.6
G-50 pool 3	2.8[c]	35.1[c]	<0.1	<0.04	0.005

[a] MED necessary to induce net secretion of water.
[b] Weight of the acetone precipitate of the broth filtrate is taken as 100%.
[c] Recovery of this fraction was insufficient for these determinations. Values cited are the same K_{av} pool, with some enterotoxic potency, after G-50 gel filtration only.

TABLE 6. *Effect of heat, acid, and proteolytic enzymes on enterotoxic activity*[a]

Treatment	Duration (h)	Retentate G-50 pool 3	Filtrate G-25 pool 2
None		−48	−52
100 C	0.5	−68	−14
pH 1	4.0	−67	−64
Pronase	24.0	−42	−71
Trypsin	3.0	−46	−61

[a] Water transport. Both GF fractions were perfused at a concentration of 10 μg/ml.

fraction. The partial disappearance of activity in the UM-2 retentate after washing suggests that the fractions on both sides of this membrane, which has a molecular size cutoff of approximately 1,000, may be the same and that the enterotoxin has a molecular weight of greater than 1,000 but a configuration that permits it to pass through this membrane. The elution characteristics of the active fractions obtained during further purification procedures by sequential gel filtration are in accord with this. After passage through a Sephadex G-25 column, the most active eluates of both UF fractions were in, or adjacent to, the void volume and the most potent fractions of these eluates after subsequent passage through a Sephadex G-50 column were present in protein peaks that elute at a K_{av} of between 0.4 and 0.6. These observations thus suggest that there is a single *Klebsiella* ST that has an apparent molecular weight in the range of 5,000. Whether the equally potent protein peak that was present in the void volume of the G-50 eluate of the UM-2 filtrate represents another separate enterotoxin or, as is probably more likely, a polymer of the larger-sized toxin is unknown. Evidence obtained by Mitchell et al. (30), who also used UF and GF separation techniques, suggests that an *E. coli* enterotoxin with a molecular weight in the range of 6,000 fragments to produce a smaller-sized toxin.

Although Gyles and Barnum found that the enterotoxic activity is not associated with cell wall or capsular preparations of *E. coli* (14), Jacks et al. in their attempt to purify the large-molecular-weight *E. coli* LT were unable to separate the enterotoxic fraction from endotoxin (18). The results of the present study indicate that *K. pneumoniae* ST is not related to the endotoxic carbohydrate-lipid-protein complex: (i) the active enterotoxin fractions eluted during G-25 GF in advance of the areas of peak carbohydrate activity; (ii) the elution characteristics of the active fractions separated by G-50 were clearly different from the endotoxin that has a large molecular weight (1) and would thus be expected to appear in the void volume; and (iii) the carbohydrate content of the most purified enterotoxin fractions is less than 3% and the protein content ranges from 35 to 43%, whereas endotoxin consists of from 40 to 50% carbohydrate and about 10% protein (31).

Although the strain of *K. pneumoniae* used in this study elaborates only ST, we have recently identified another strain of this species, isolated from a Haitian with tropical sprue, which produces both ST and LT (24). *K. pneumoniae* thus appears to resemble *E. coli* in that certain strains elaborate ST alone and others produce both ST and LT (13, 36). Attempts at purifying and defining the precise molecular weight of the *E. coli* enterotoxins has met with limited success. UF procedures indicate that *E. coli* LT has a molecular weight in excess of 100,000 (18, 26). UF and GF studies by Mitchell et al. (30), working with a broth filtrate of *E. coli* whose heat lability was not defined, suggest that several toxic fractions are present whose molecular weight varies from 5.9×10^5 to 6,700. Similar studies by Lariviere et al. (26) and by Evans et al. (7) also indicate that *E. coli* LT exists in numerous large molecular weight forms. In contrast, *E. coli* ST is similar to *K. pneumoniae* ST in that it has a low molecular weight. After sequential passage of an acetone precipitate of an *E. coli* ST through UM-10 and UM-2 UF membranes, Bywater found activity only in the UM-2 retentate and filtrate fractions (2) and studies of a similar nature by Jacks and Wu (19) also led them to the conclusion that the molecular weight of *E. coli* ST is in the range of between 1,000 and 10,000.

The responses of *K. pneumoniae* ST to various physical, chemical, and enzymatic treatments are also similar to those of *E. coli* ST. Exposure to a temperature of 100 C for 30 min reduces, but does not abolish, the activity of crude preparations of *E. coli* ST (6, 25, 33, 36) although Jacks and Wu found that such treatment did abolish the activity of an *E. coli* ST preparation that had been paritally purified by UF and GF procedures (19). The activity of the purified GF fraction of the *K. pneumoniae* ST UM-2 filtrate was not affected at all by heat treatment, whereas that of the GF fraction of the retentate was reduced but not abolished. *E. coli* LT is labile to mild acid treatment (25, 30), but ST is resistant to pH values as low as 1 (19, 25); so is *K. pneumoniae* ST. Pronase treatment abolishes the activity of *E. coli* LT (18) but has no effect on either *E. coli* ST (19) or *K. pneumoniae* ST. Trypsin treatment does not affect either *E. coli* LT or ST (14, 18, 25) or *K. pneumoniae* ST.

These observations indicate that the low-mo-

lecular-weight, heat-stable enterotoxins elaborated by *E. coli* and *K. pneumoniae* bear many similarities. It remains to be determined, however, whether the low-molecular-weight toxins produced by these two species, as well as by other species of coliform bacteria such as *E. cloacae* (F. A. Klipstein, and R. F. Engert, unpublished observations), represent a single, chemically and physically homogeneous toxin. Crude preparations of ST are not antigenic (5, 6, 36); however, the antigenicity of purified preparations has not been tested so it is unknown whether, if they are antigenic, they share the same properties.

ACKNOWLEDGMENTS

We thank Michael D. Turner for his advice concerning the gel filtration studies.

This work was supported by grants from the Williams-Waterman Fund of the Research Corp., New York City, the Hillsdale Fund, Greensboro, N. C., and Public Health Service training grant 5-TO-1-AM-05177 from National Institute of Arthritis, Metabolism and Digestive Diseases.

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