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Assay, Characterization, and Localization of an Enterotoxin Produced by *Salmonella*

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An enterotoxic factor isolated from cultures of *Salmonella* yielded reproducible results in the suckling mouse model in contrast to other animal models. The enterotoxin appears to possess properties similar to both the heat-stable and heat-labile enterotoxins of *Escherichia coli*. Preliminary results indicate that the toxin is a protein, is located in the cell wall or outer-membrane fraction, and is difficult to separate from other cell wall constituents.

In the recent past significant progress has been made in understanding enteric disease associated with *Vibrio cholerae* and enteropathogenic *Escherichia coli* (10). The cholera toxin has been identified as a protein and has been crystallized (8) and partially characterized (15). However, progress with the heat-labile toxin (LT) of *E. coli* has been hampered due to difficulties in separating the toxin from the lipopolysaccharide (LPS) endotoxin (14).

Despite the fact that salmonellosis accounts for a significant amount of enteric disease, comparatively little effort has been made to enhance our understanding of the pathogenic mechanism(s) associated with *Salmonella*. In this communication we report the finding of an enterotoxic principle that appears to be associated with the cell wall or outer membrane of *Salmonella enteritidis*.

MATERIALS AND METHODS

Cultures. The cultures used in this study were obtained from E. S. H. Christenson, Wisconsin State Laboratory of Hygiene, Madison. All cultures were isolated from relatively recent clinical cases, but clinical histories of the patients were not available. In the course of this study the cultures were transferred many times and no attempt was made to stock the original culture. All of the experiments regarding purification of the toxin involved one strain of *Salmonella, S. enteriditis* serotype 1,9,12:g,m.

In certain phases of the study, a non-enteropathogenic *E. coli* strain was included for control purposes. This culture served as a control to indicate the possible carry-over into the mouse assay of reagents used in the purification procedures.

Media and maintenance of cultures. The cultures were maintained by daily transfer in brain heart infusion (BHI) broth. In the early phase of the study, the experimental cultures were grown in BHI; in the later phase, the syncase medium described by Finklestein et al. (7) was modified by substituting an equal concentration of glucose for sucrose.

Incubation. All cultures were incubated at 37 C. The experimentally grown cultures (1 liter in a 2.8-liter Fernbach flask) were incubated on a gyratory shaker set at 180 rpm.

Toxigenicity could not be readily demonstrated in young cultures by the mouse assay (absorbance <0.5) or in cultures in which the absorbance exceeded approximately 1.4. Consequently, during growth the absorbance was monitored and the cultures were harvested when the absorbance was in the range of 1.1 to 1.4.

Assays for enterotoxic activity. The ileal loop procedure described by Sack et al. (23) was used in this study to screen for enterotoxic activity in various animals. The suckling mouse assay described by Dean et al. (2) was slightly modified as indicated in Results. Activity in this assay is expressed as a ratio of the intestinal weight to the remaining total body weight, and, in our study, any value greater than 0.08 was considered a positive response. In the early phase of the study, we used only the ratio. In the later phase, it was deemed desirable to equate the activity with the protein concentration in various preparations. We define this as the specific enterotoxic activity and calculate it as follows:

$$\frac{\text{intestinal weight/body weight} - 0.08}{\text{milligrams of protein/dose}} = \text{specific enterotoxic activity}$$

In our procedure, three mice were used for each preparation or concentration of toxin assayed and the results were averaged. In any given trial, minimal variation among the animals was noted. However, some variation in the preparations was observed regardless of attempts to grow the organism under identical conditions. Rarely, *S. enteriditis* cultures completely devoid of enterotoxic activity were observed.

Culture supernatant fluid. Throughout this study various culture supernatant fluids were examined for

enterotoxic activity. In most instances, concentration was achieved by ammonium sulfate fractionation wherein all precipitable material at 80% saturation (2 to 4 C) was centrifuged and the pellet was dialyzed against two changes of phosphate buffer (0.05 M; pH 7.0).

Enzymes. The following enzymes were used to test their effect on crude preparations of the enterotoxin: Pronase (B grade, Calbiochem); bacterial alpha-amylase, type II-A; trypsin, type XI; lysozyme, grade 1; and phospholipases A, C, and D (all from Sigma Chemical Co.).

Density gradient centrifugation. All density gradient centrifugations were conducted at 4 C with an SW-41 rotor in an L265-B preparative ultracentrifuge (Beckman Instruments). After centrifugation, the density gradient tubes were removed and fractionated either by puncturing the bottom of the tube or by removing the gradient with a fine capillary tube and a peristaltic pump. One-milliliter fractions were collected while the protein concentration was monitored with a Uvicord II ultraviolet light absorptiometer (LKB Instruments) set at 280 nm.

Chemical analyses. Protein estimations were made by the method of Lowry et al. (16), with lysozyme as the standard. The total cytochrome content of the membrane fractions was estimated by the procedure described by Hager and Deeb (13). The membrane fractions were suspended in 10 mM tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.8; protein concentration, 1 to 10 mg/ml). Sodium deoxycholate was added to a final concentration of 2.0%, and the sample was incubated for 15 min (24 C) before centrifugation at 10,000 $\times$ *g* for 10 min. The supernatant fluid was decanted and added to cuvettes. Nothing was added to the reference cuvettes; a few crystals of dithionite were added to the sample cuvettes (20). Difference spectra were estimated by using a Cary 15 recording spectrophotometer (Applied Physics Corp.). Heptose concentrations in the membrane fractions were estimated by the cysteine-hydrochloride reaction (4) as modified by Osborn (19). Glucoheptose (Sigma) was used as the standard (3). Dry weights of the membrane fractions were estimated after exhaustive dialysis against distilled water and subsequent lyophilization. The samples were stored for 2 days over Drierite before weight determinations.

Estimations for enzyme activities. Succinate and lactate dehydrogenase activities and reduced nicotinamide adenine dinucleotide (NADH) oxidase in the membrane fractions were estimated by the methods outlined by Osborn et al. (20). The specific enzyme activities are expressed as the change in absorbance per minute per milligram of protein.

RESULTS

Study of the suckling mouse assay system. Initial studies using ileal loops in the rabbit, guinea pig, rat, and chicken models yielded inconsistent results with various *Salmonella* serotypes. Similar results have been reported with other enteric pathogens (17, 22). In our early experiments, fully grown BHI cultures (incubated for 18 to 24 h at 37 C aerobically) were used as the inoculum. In subsequent experiments it was observed that exponential-phase cultures gave significantly more reproducible results. A survey of five *Salmonella* serotypes indicated that *S. enteritidis* yielded the most reproducible results and that it was the most sensitive in the rabbit model. This procedure, however, is extremely laborious, and attempts to utilize the suckling mouse assay system proposed by Dean et al. (2) indicated that it was more accurate and sensitive as well as less laborious as compared with the rabbit ileal loop technique. The majority of the subsequent experiments used the mouse model and culture supernatant fluids (see next section) from exponential-phase cultures. It was observed that animals 3 to 5 days old were ideal and other animals (6 to 9 days old) evidenced decreasing sensitivity to the toxic principle.

Early experiments indicated that the time of incubation of the inoculated animals was of import, and several experiments verified an optimal incubation period of 2.5 h (Table 1). This incubation period was used throughout the study. These results are in contrast to the 4-h incubation period recommended by Dean et al. (2) for the assay of enteropathogenic *E. coli*.

Activity comparison of whole cultures, culture supernatant fluids, and washed cells. An attempt was made to ascertain the relative activity of culture supernatant fluids and washed cells of *S. enteritidis* and *S. typhimurium*. Aerobically grown cultures in BHI were chilled on ice after the absorbance reached a value of 1.4. Ten milliliters of culture was centrifuged, the supernatant fluid was collected, and the cells were washed once and resuspended in saline. The pH value of all preparations was adjusted to 6.5, and immediately before assay in the mice, the preparations were tempered in a 37 C water bath. All prepa-

TABLE 1. *Effects of incubation time on the suckling mouse assay using S. enteritidis culture supernatant fluids*

Incubation period (h)	IW/BW ratio[a]	
	Trial no. 1	Trial no. 2
1	0.070	
2	0.087	0.091
2.5	0.100	0.120
3	0.092	0.096
4	0.078	0.081

[a] Intestinal weight/remaining body weight ratio.

rations gave a positive result but characteristically, in many trials, the supernatant fluid possessed the highest activity (Table 2). Consequently, in the majority of the subsequent experiments, culture supernatant fluids were used as the source of the enterotoxin.

Effect of heating and freezing. Two types of enterotoxin are produced by *E. coli;* they are differentiated primarily by their reactivity after heating and secondarily by their differential response in ileal loops. The heat-stable toxin (ST) is relatively stable at 100 C but is destroyed by autoclaving (26). The LT toxin is inactivated by heating to 60 C for 30 min, but some variations exist depending upon strain involved.

The *Salmonella* concentrated culture supernatant (60 mg/ml of protein) was subjected to decimal increments of temperature from 50 to 100 C for 30 min. At temperatures of 50, 60, and 70 C, the toxin was stable, but all activity was destroyed at 80, 90, and 100 C. In this respect the *S. enteritidis* toxin resembled the LT of *E. coli*. In another series of experiments it was observed that the toxin was stable to freezing (−20 C), although a slight decrease in activity was noted.

Effect of pH value. Evans et al. (6) have demonstrated that the LT of *E. coli* is sensitive to acidic pH values but not to mild alkaline conditions. In contrast, the ST is refractory to both alkaline and acidic conditions. To test the pH stability of the *S. enteritidis* toxin, the concentrated supernatant fluid was added to various buffers, and the pH value before and after incubation at 24 C for 24 h was estimated. After incubation, the solutions were neutralized, the volumes were adjusted (final protein concentration was 15 mg/ml), and the test solutions were assayed for enterotoxic activity. The toxin was stable at all pH values tested, and in this respect it resembles the ST of *E. coli* (Table 3).

Effect of various enzymes on the toxic principle. Most enterotoxins are protein in nature and, as might be expected, they are unaffected by treatment with trypsin and a variety of other hydrolytic enzymes. Pronase, however, generally destroys all enterotoxic activity. To gain more information regarding the chemical nature of *S. enteritidis* factor, samples of a concentrated culture supernatant fraction (60 mg/ml of protein in the final test solution) were incubated individually with the following enzymes: Pronase, lysozyme, trypsin, amylase, and phospholipases A, C, and D. All preparations were incubated for 3 days. In keeping with other enterotoxic principles, the *S. enteritidis* enterotoxin was inactivated by Pronase and unaffected by the other enzymes tested (Table 4).

Association of enterotoxic activity with the cell membranes. The observation that the active principle was associated with a particulate fraction and not excreted as an exotoxin is analogous to the observations made by many investigators working with the LT of *E. coli*. An

TABLE 2. *Relative activity of S. enteritidis and S. typhimurium cultures in the suckling mouse assay*

Organism	IW/BW ratio[a]		
	Whole culture	Culture supernatant fluid	Washed cells
S. enteritidis	0.104	0.113	0.94
S. typhimurium ..	0.110	0.119	0.113
BHI control	0.080		

[a] Intestinal weight/remaining body weight ratio.

TABLE 3. *Effect of pH value on stability of the S. enteritidis enterotoxin*

pH value		IW/BW ratio[a]
Before incubation	After incubation	
2.0	1.9	0.131
4.2	4.1	0.133
6.0	6.2	0.119
7.3	7.3	0.142
8.1	7.9	0.131
10.2	9.9	0.134
Buffer controls		<0.080

[a] Intestinal weight-remaining body weight ratio.

TABLE 4. *Effect of various enzymes on the S. enteritidis enterotoxin*[a]

Enzyme tested	Enzyme concn in test solution (mg/ml)	Incubation temperature (C)	IW/BW ratio[b]
None		24	0.101
None		37	0.105
Pronase	0.05	37	0.079
Trypsin	2.50	24	0.112
Amylase	2.50	37	0.101
Lysozyme	0.10	37	0.097
Phospholipase A .	2.5	37	0.110
Phospholipase C .	2.5	37	0.100
Phospholipase D .	2.5	24	0.095

[a] For details of test procedure see text.
[b] Intestinal weight/remaining body weight ratio.

extended effort was made to obtain more definitive information regarding this supposition in regard to the *Salmonella* enterotoxin.

To avoid the complications of using a complex medium for the growth of *S. enteritidis*, a modified syncase medium was used (see Materials and Methods). This medium afforded good toxin production and was used in all subsequent experiments. To prepare the membrane fractions, 1 liter of the modified syncase medium was inoculated with a washed culture of *S. enteritidis* and incubated aerobically until an absorbance of 1.1 at 640 nm was reached. The cells were sedimented by centrifugation and the supernatant fluid was saved for further analyses. The cell pellet was washed with 0.05 M Tris buffer (pH 7.8) and ultimately resuspended in 200 ml of Tris buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA). After blending in an Eberbach jar for 1 min, the preparation was centrifuged and the supernatant fluid was decanted and saved (EDTA extract). The pellet was resuspended in Tris buffer (pH 7.5), and then 1.0 mg of ribonuclease and 1.0 mg of deoxyribonuclease were added before cellular disruption in a French pressure cell (two treatments at maximal pressure). Most of the cells were ruptured by this treatment as observed visually under a phase-contrast microscope. Sufficient $MgCl_2$ was added to obtain a 2 mM Mg^{2+} concentration, and the preparation was incubated at ambient temperature for 0.5 h. This was followed by centrifugation (3,000 × *g*) for 5 min to remove whole cells, and the supernatant fluid was then centrifuged at 120,000 × *g* for 1 h. The supernatant fluid (Mg^{2+} extract) was decanted and the pellet was resuspended in 10 ml of Tris buffer containing 0.4 mg of Lubrol PX (Sigma Chemical Co.). After 5 min of incubation at 20 C, the preparation was centrifuged (120,000 × *g*) and the supernatant fluid was collected (Lubrol extract). The pellet was resuspended in distilled water and a portion was treated by the *N,N*-dimethyl formamide technique described by Schnaitman (24). The 280 nm-absorbing fraction was collected and dialyzed for 24 h against two changes of 0.05 M Tris buffer. The dialysate was concentrated to 10 ml by using a Diaflow PM-10 membrane. Chemical estimations for protein and enterotoxic activity were determined on all extracts.

The EDTA treatment released a significant amount of activity, the magnesium chloride released about one-half as much, and the Lubrol was essentially ineffective (Table 5). The *N,N*-dimethyl formamide treatment, which effects the separation of lipid and protein in *E. coli* membranes, liberated a significant amount of the enterotoxic activity. However, the activity of the preparation rapidly diminished after 24 h at 4 C. The results indicated that some of the toxin is loosely associated with the cell but the majority is strongly bound in either the inner or outer membrane.

TABLE 5. *Relative amounts of enterotoxic activity located in various fractions of S. enteritidis cultures*

Fraction	Total protein (mg)	Protein/dose of dilution tested (μg)	Specific enterotoxic activity
Culture supernatant fluid	400	4.4	9.1
EDTA extract	60	3.0	16.5
$MgCl_2$ extract	175	3.5	7.2
Lubrol extract	250	25.0	0.8
Membrane pellet	98	9.8	2.9
DMF-extracted membrane[a]	65	0.7	57.0

[a] DMF, *N,N*-dimethyl formamide.

To preclude any possible carry-over of reagents used in the purification procedure, a non-enteropathogenic *E. coli* culture was treated identically to that of the *S. enteriditis* strain. Each *E. coli* fraction, identical to those listed in Table 5, failed to elicit any activity in the mouse assay.

Discontinuous density gradient centrifugation. Earlier experiments indicated that the active principle was particulate in that the activity could not be filtered through a XM-100 membrane and it always eluted in the void volume of a Sephadex G-100 column. Similar observations with the *E. coli* toxin were reported by Jacks et al. (14). In an effort to obtain more definitive information regarding this observation, the concentrated culture supernatant fluid as well as a concentrated cell extract (made by resuspending a whole-cell pellet in 1 mM EDTA followed by centrifugation) were subjected to a discontinuous density gradient centrifugation procedure (25). Three concentrations of sucrose were used for separation of the particles (2.02, 1.44, and 0.77 M in 0.05 Tris and 1 mM EDTA, pH 7.8). After addition of the two test solutions to separate tubes, the preparations were centrifuged at 25,000 rpm for 16 h. The fractions were collected by removing the gradients with a peristaltic pump; as each fraction was removed, it was monitored for protein by scanning at 280 nm (Fig. 1 and 2). The fractions were then freed of sucrose by dialysis against two 6-liter changes of a solution consist-

ing of 0.05 M Tris buffer containing 5 mM EDTA. The dialysis was conducted at 4 C over a 24-h period. After dialysis and concentration,

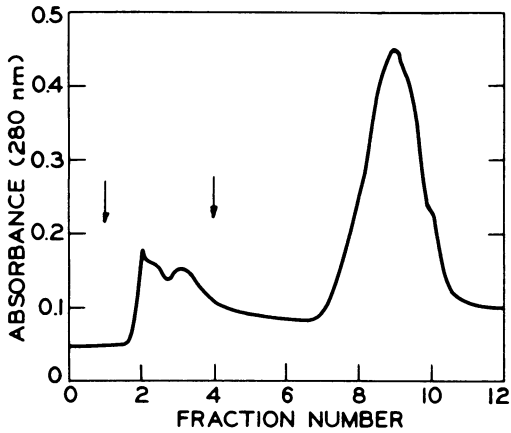

FIG. 1. *Sucrose density gradient centrifugation of the concentrated culture supernatant fluid from S. enteritidis. Experimental procedure is as described in text. Decreasing density of the fractions is left to right. Arrows indicate range of fractions in which enterotoxic activity was found.*

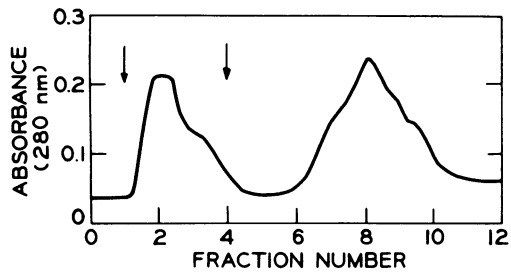

FIG. 2. *Sucrose density gradient centrifugation of the concentrated EDTA extract from S. enteritidis cells. Experimental procedure is as described in text. Decreasing density of the fractions is left to right. Arrows indicate range of fractions in which enterotoxic activity was found.*

a chemical analysis for total protein and heptose (characteristic of the heavy or outer membrane fraction) was performed and all fractions were assayed for enterotoxic activity.

In both the culture supernatant fluid and EDTA extract samples the enterotoxic activity sedimented with the heavy particles (Table 6). In both fractions there was a diffuse band of light particles and a relatively dense band of heavy particles. These observations focused attention on the cell surface or outer membrane of the cell and led to attempts to free the activity from the cell as will be described in the following section.

Separation of inner and outer membranes. An attempt was made to localize the *Salmonella* enterotoxic activity to either the heavy (outer) membrane or the light (inner or cytoplasmic) membrane. After several preliminary trials, it was observed that the spheroplasting procedure described by Osborn et al. (20) appeared to be ideally suited for our purpose. Essentially, this procedure was followed except for the density gradient centrifugation for the separation of the membranes. Our goal was to effect as sharp a separation as possible between the outer and inner membranes, and the procurement of subfractions was not necessary. Consequently, the sucrose concentrations were altered as follows: 3 ml of 2.02 M sucrose was dispensed to the centrifuge tube followed successively by the layering of 4.0 ml of 1.44 M and 4.0 ml of 0.77 M sucrose. One milliliter of the membrane fraction (washed membranes were resuspended in 10 ml of 0.05 M Tris-5 mM EDTA-0.25 M sucrose) was layered over the latter and the preparation was centrifuged at 25,000 rpm for 16 h as described previously. The gradients were removed by puncturing the bottom of the tubes and fractions of 1.0 ml were collected and monitored for protein at 280 nm. After combining the fractions and dialysis, the light and heavy fractions were assayed for total cytochrome content,

TABLE 6. *Density gradient centrifugation of culture supernatant fluid and an EDTA extract of whole cells*

Sample	Fraction	Specific enterotoxin activity	Heptose (μg/mg [dry wt])	Protein (mg/mg [dry wt])
Culture supernatant fluid	Light particles	0	NT[a]	NT
	Heavy particles	3.8	2	0.02
	Not centrifuged	4	2	0.06
EDTA extract	Light particles	0	NT	NT
	Heavy particles	25	NT	NT
	Not centrifuged	17	26	0.49

[a] NT, Insufficient quantity for testing.

NADH oxidase, succinate and lactic dehydrogenase activities, total protein, heptose, and dry weight.

In the sucrose gradient, two sharp and distinct bands were observed. The majority of the enterotoxic activity was confined to the heavy particles (outer membrane, Fig. 3). After combining and dialyzing the fractions, their cytochrome, heptose, and protein contents were estimated (Table 7). The light-membrane fraction contained the majority of the cytochrome pigments, whereas the heavy-membrane fraction contained the majority of the heptose. Although the heavy-membrane fraction contained some NADH oxidase and lactic and succinate dehydrogenase activities, most of the enzymatic activity was associated with the light membrane fraction. As before, most of the enterotoxic activity was found in the heavy-particle fraction (Table 8).

Although the enzymatic activities and the heptose were not clearly delineated to the membrane fractions with which they are commonly associated, the preponderance of the activities could be definitively associated with their respective fractions. Several possible explanations exist to account for lack of a sharp separation. The method used in this experiment of obtaining the fractions from the sucrose gradient tubes (puncturing the tube and allowing the fractions to flow out the bottom) could have led to some contamination of the light- or inner-membrane fraction. Alternatively, smaller particles of the outer membrane could sediment with the inner membrane. Similar results were observed in a previous study (20).

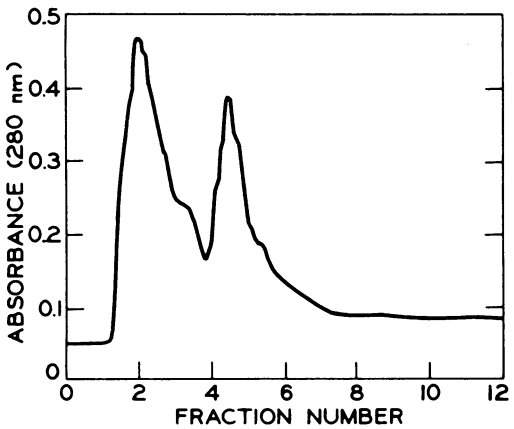

FIG. 3. *Sucrose gradient centrifugation of total membrane fraction from S. enteritidis. The total membrane fraction was prepared and subjected to sucrose density gradient centrifugation as described in text. Decreasing density of the fractions is left to right.*

TABLE 7. *Composition of membrane fractions*

Fraction	Cytochrome (mM/mg)	Heptose (mg/mg [dry wt])	Protein (mg/mg [dry wt])
Total membrane	0.0012	0.033	0.33
Light membrane	0.0026	0.008	0.13
Heavy membrane	0.0009	0.022	0.27

TABLE 8. *Localization of enzyme activities*

Enzyme	Sp act[a]		
	Total membranes	Light membranes	Heavy membranes
NADH oxidase	4.55	13.3	0.85
Lactate dehydrogenase	1.14	3.8	0.94
Succinic dehydrogenase	0.51	0.8	<0.09
Enterotoxin	690	160	560

[a] Expressed as change in absorbance per minute per milligram of protein except for enterotoxin, which is expressed as stated in Materials and Methods.

Effect of chloroform and phenol extractions. The cell crop of 1 liter of modified syncase broth was harvested and EDTA, ribonuclease, and deoxyribonuclease were added in the previously described manner before passing the preparation through a French pressure cell. After centrifugation to remove the intact cells, $MgCl_2$ and Lubrol were added and the preparation was centrifuged at 134,000 × *g* for 90 min. The pellet was resuspended in Tris buffer and constituted the washed-membrane fraction.

Free lipid was extracted from the membranes by the chloroform-methanol-water procedure described by Osborn et al. (20). Two subfractions were obtained from this step: an aqueous and an organic extract. A portion of the aqueous phase was extracted by the phenol procedure described by Westphal and Jann (29). Two additional fractions were obtained: a phenol extract and the residual aqueous extract. For comparative purposes, the culture supernatant fluid was concentrated and subjected to the phenol extraction procedure. After dialyzing the extracts, the protein concentration and enterotoxic activity were estimated.

The chloroform extraction procedure did not destroy the enterotoxic principle (Table 9). Indeed, this procedure aided in increasing the specific enterotoxic activity; however, the activity rapidly diminished after 24 to 48 h of storage at 4 C. The phenol extraction procedure completely destroyed all enterotoxic activity in both

TABLE 9. *Effect of various extraction procedures on the enterotoxic activity of S. enteritidis membranes and concentrated culture supernatant fluid*

Sample	Protein (mg/ml)	Specific enterotoxic activity
Untreated membranes	14.0	1.2
Chloroform extraction		
Organic extract	NT[a]	0
Aqueous extract	0.6	20.0
Phenol extraction		
Phenol extract	0.87	0
Aqueous extract	0.50	0
Phenol extraction (concentrated culture supernatant)		
Untreated	0.45	4
Phenol extract	0.25	0
Aqueous extract	0.09	0

[a] Not tested.

the membrane fraction and the culture supernatant fluid. These results further negate the possible involvement of the LPS in eliciting intestinal fluid accumulation. Other investigators (12, 18, 27) have also reported that LPS preparations from other organisms did not provoke intestinal fluid accumulation.

DISCUSSION

Although the suckling mouse assay has been reported as being insensitive to the LT of *E. coli* and the *V. cholerae* enterotoxin (CE), it appears to be quite sensitive to the *Salmonella* enterotoxin (SE). More sensitive and reproducible results were obtained with this model as compared with ileal loop models including the infant rabbit. The suckling mouse assay is also less expensive and time consuming, and a larger number of trials are possible with a given amount of enterotoxin preparation.

Reducing the incubation time of the inoculated mice from 4 to 2.5 h and using 3- to 5-day-old mice significantly increased the sensitivity of the assay. Another factor relating to sensitivity of this assay was observed when concentrated enterotoxin preparations were used. In many instances, poor or negative results were observed when highly concentrated preparations were injected. However, dilution of the preparation afforded a quantitative response. This is somewhat reminiscent of the prozone phenomenon in the precipitin reaction.

No explanation can be advanced at this time regarding the failure of the suckling mouse assay to respond to LT or to CE (2). This may reflect acid sensitivity or sensitivity to degradation by pepsin. This assay is certainly quite sensitive to SE, which may reflect a basic difference in the mechanism of action of the different toxins.

Many similarities between LT and CE have been observed by various investigators (see review by Gyles, 11; 28). Although the SE evidences some differences with these toxins, it also possesses some common properties. Both the LT and the CE have been shown to be inactivated completely by heating to 60 C for 30 min. In comparison, the SE was somewhat more heat stable in that inactivation did not occur until a temperature of 80 C (for 30 min) was reached. The comparison with the LT may not be valid, since there appears to be a great deal of confusion regarding the thermostability of the toxin (14). In regard to the ability of SE to withstand freezing, it appears to be similar in its resistance to both the LT and the CE. All three toxins also exhibit similar properties in their stability to trypsin and sensitivity to Pronase. These results suggest that all three toxins have a protein component that is necessary for the manifestation of enterotoxic activity.

Some differences between the LT, CE, and SE are quite evident. The stability of the LT to acid is reported to be poor, whereas both the LT and the CE are stable under alkaline conditions. The SE appears to be resistant to both acid and alkali, and its resistance to acid may have some significance in regard to food poisoning since it can withstand the acidity of the stomach. However, its resistance to pepsin is yet to be tested. Apparently, the SE shares properties with both the LT and the ST. It is similar to the ST in regard to pH stability and reactivity in the mouse assay, and it is similar to the LT in regard to heat stability and in that crude preparations are nondialyzable, eluted in the void volume in a Sephadex G-100 column, and retained by a XM-100 Diaflow membrane.

Recently, Donta and Smith (5) reported that culture supernatant fluids from a variety of enterotoxin-producing organisms (including an unknown number of *Salmonella* sp.) were inactive in the adrenal cell, tissue culture model. Although further study is warranted, the observation indicates another difference in the comparison of LT and SE. A tabular comparison of SE with LT and ST suggest that the SE shares certain properties with both LT and ST (Table 10). However, there are significant differences, and the possibility that a common plasmid can account for the enterotoxic activity shared by *V. cholerae*, *E. coli*, and *Salmonella* does not appear to be warranted at this time in spite of

TABLE 10. *Comparison of various properties of the heat-labile (LT) and heat-stable (ST) toxins of E. coli with the enterotoxin of S. enteritidis (SE)*

Enterotoxin	Assay model[a]		Heat resistance			Stability to:		Dialysis
	Suckling mouse	Adrenal cell	60 C	80 C	100 C	Acid	Alkali	
LT	–[b]	+[c]	–	–	–	–	+	Not dialyzable
ST	+	–	+	+	+	+	+	Dialyzable
SE	+	–	+	–	–	+	+	Not dialyzable

[a] – Indicates a negative response in the assay model or inactivation under the physical condition under test. + Indicates a positive response in the assay model or resistance to the physical condition under test.

[b] Response of the suckling mouse to LT has not been established definitively (W. B. Greenough, personal communication).

the fact that this assumption is probably valid for *V. cholerae* and *E. coli* (9).

The origin of the SE appears to be in the membrane portions of the cell. This may not be immediately evident unless some of the lipid is removed by organic solvents or liberated by treatment with *N,N*-dimethyl formamide. After these treatments, significant increases in the specific enterotoxic activity were observed. The cellular location of the SE parallels data obtained by Gyles (12) in his studies with *E. coli*. Using the pig ileal loop model, it was observed that if whole-cell preparations reacted positively, the corresponding cell lysates elicited a positive response. Also, whole cells from strains that reacted negatively yielded lysates that failed to produce a positive response. Similar results were reported earlier by Moon et al. (18), using the rabbit model. Aside from these observations, the cellular location of both LT and ST is unknown. However, suggestive data relating a close chemical relationship of LT with LPS is well documented (14). Although the release of CE is accompanied by significant amounts of LPS, it is not apparently bound to the same extent as the LT and LPS in *E. coli* even though the LPS of *V. cholerae* does block the reactivity of antitoxin to some extent (21).

The separation of the membrane fractions of *Salmonella* into an enriched outer-membrane fraction (H-membrane) and an enriched inner-membrane fraction (M_1-, L_1-, L_2-membranes) by the discontinuous density gradient technique has demonstrated that the majority of the intercellular enterotoxic activity is associated with the outer-membrane fraction. Some activity was localized in the inner-membrane fraction that was probably due to technical manipulations or to an incomplete separation of the two fractions. This was evidenced by the fact that heptose, a component of LPS, was found in low concentrations in the inner-membrane fraction. The M-membrane, as reported by Osborn et al. (20), has both LPS and some cytoplasmic membrane enzyme activity. The specific density of the M-membrane is less than that of the H-membrane but slightly more than the density of the L-membranes. Our step gradients did not distinguish the M-band and it could have sedimented with the inner-membrane fraction, thus accounting for some enterotoxic activity. Previous experiments with the concentrated culture supernatant fluid and with EDTA extracts demonstrated that the enterotoxic activity of these preparations was localized in a band corresponding to that density in which the outer membrane would be found. Heptose determinations reinforced this contention and indicated that the active material in both the supernatant fluid and the EDTA extract of whole cells originates from the outer membrane of *Salmonella* and that it is released during growth.

The question of endotoxin being associated with enterotoxic activity led us to test LPS extracts from *Salmonella* for activity in the mouse assay. Previous investigations with *E. coli* demonstrated that endotoxin, prepared by extraction with phenol and water, did not provoke a positive reaction (12, 18, 27). Extraction of a membrane fraction and a culture supernatant fluid preparation from *Salmonella* by the phenol-water procedure completely destroyed all enterotoxic activity. Thus, the enterotoxin of *Salmonella* is similar to the LT of *E. coli* in that the activity is associated with but is not endotoxin per se.

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