INVESTIGATION OF 'E. coli' ENTEROTOXINS

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ABSTRACT

This report summarizes research performed under USAMRIID Contract #DADA17-74-4007 during the period from August, 1973 through May, 1974. The data describes our progress in the areas of toxin production, assay and purification. One of the major aspects of our work has been the demonstration that E. coli enterotoxin(s), like cholera toxin, cause vascular permeability changes in rabbit skin. This observation has permitted us to apply the rabbit skin test developed for cholera toxin by Dr. John P. Craig to the measurement of E. coli heat-labile enterotoxin. By using this assay, it has been possible to investigate a variety of growth conditions and to select from these a set of conditions (a modified syncase medium supplemented with yeast extract; 24 h shaker cultures; 37°) which have been used routinely to produce enterotoxin for purification studies. At the same time, the availability of the assay has permitted the screening of a number of drug-resistant variants for increased toxin production. On the basis of vascular permeability activity, it has been determined that certain drug-resistant variants do indeed produce greater amounts of enterotoxin than conventional enterotoxigenic strains. Taken together, these findings have measurably improved the outlook with respect to solving the problems of easy assay and higher enterotoxin yields.

To date, the bulk of our purification studies have been performed using culture filtrates produced by enterotoxigenic strain 197. The results have shown that the enterotoxin can be concentrated quantitatively by ammonium sulfate precipitation and further purified by a series of high speed sedimentations. The fact that the enterotoxin could be separated from the bulk of soluble proteins by sedimentation indicated that it was a molecule of considerable molecular weight. Further studies revealed that the functional enterotoxin could be broken down into a smaller molecule of about 40,000 to 50,000 M.W.; but a complete description of this conversion has not yet been achieved. While substantial purification of the enterotoxin has been achieved by repeated sedimentation, its complete purification has been hindered by the co-sedimentation of other high molecular weight contaminants, some of which appear to be somatic antigens.

The details of our findings are presented in the following report.

I. Production of Enterotoxin

A. Rabbit Skin Vascular Permeability Data

Data summarizing the production of enterotoxin by E. coli strains 408-3 and 197 are given in Table I. The organisms were grown either in syncase medium or in a medium supplemented with 0.6% yeast extract (Evans, Evans and Gorbach, Infect. & Immun. 8:725-730, 1973). The latter medium is designated in this report as syn/yeast medium. Unless specified otherwise, the organisms were harvested by centrifugation after incubation at 37° for 24 hr. The culture supernatants were filtered aseptically through 0.45 micron Millipore filters and then assayed for vascular permeability activity in the rabbit skin test. The amount of enterotoxin contained in the culture filtrates was based on the dilution of toxin which yielded a 4 mm blueing lesion 18 hr after intradermal injection of 0.1 cc.

Although a direct comparison of toxin produced by strains 408-3 and 197 was carried out in only two experiments (408-3-7 and 197-1; 408-3-8 and 197-12), the data compiled in a series of experiments indicates that strain 197 may produce about 3- to 4-fold more toxin than strain 408-3, under the particular set of conditions employed (compare for example, lots 408-3, 5-7 and lots 197, 1-3). In addition, the data suggest that the presence of the yeast extract in the medium further increases toxin production (compare 408-3, 1, 3 and 4 with 408-3, 5, 6 and 7; also 197-17A and 197-17B). Still another increase in toxin production was observed when the organisms were harvested at 48 hr instead of 24 hr. In one instance (408-3-2), the organisms were incubated at 37° for 48 hr; and in another instance (197-14), the organisms were incubated at 37° for 24 hr and held at 4° for 24 hr before harvesting. In the first case, increased toxin production appeared to be associated with significant lysis of the culture. In the second case, less lysis was observed, but a repetition of the experiment (197-15A and 197-15B) did not result in improved toxin yield.

On the basis of the data given in Table I, strain 197 was chosen for routine production of enterotoxin. The amount of toxin generally produced by strain 197 in Syn/yeast medium was on the order of 100-200 BD_{4mm}/ml which, on the basis of neutralization tests (see Part II), corresponds to about 1 Lb (0.05 μg) of cholera toxin. (One Lb of cholera toxin is equivalent to about 2000 BD_{4mm}.) This amount of (E. coli) toxin represents 100- to 400-fold less vascular permeability activity than found in representative Vibrio cholerae culture filtrates. In a few instances, the amount of toxin produced by strain 197 in syn/yeast medium (24 hr, 37°) was about 10-fold greater than usual (Table I, 197-8, 197-20). The reason for this is not understood. Further work on the growth requirements for maximal toxin production may lead to improved toxin yields not only with the strains presently available, but also with selected high enterotoxin-producing strains (see Part I, C).

B. Ileal Loop Data

Some of the culture filtrates for which data is presented in Table 1 were also tested in the rabbit ileal loop assay. Table 2 gives some of the data which was obtained in a series of tests. The first four tests in the Table (#2, #3, #6 and #7) show the results of our early experiments in which we used strain 408-3 and either syncase or peptone for production of enterotoxin. Although the first two tests indicated good toxin production in syncase and slightly higher production in peptone (tests #2 and #3), the results were not duplicated in subsequent batches (tests #6 and #7).

In later studies, syncase medium was supplemented with yeast extract (0.6%) (Evans, Evans and Gorbach, Infect. & Immun. 8:725-730, 1973) and a comparison of strains 197 and 408-3 indicated that 197 produced more enterotoxin (test #8), at least under the conditions employed. Further studies with strain 197 and yeast-supplemented medium (syn/yeast) yielded reasonably reproducible results (tests #8, #9, #11 and #12). In one experiment, toxin production by strain 197 was compared in syn/yeast and syncase alone. The results (test #11) suggested that the presence of yeast extract improved toxin production. It is interesting to note that the results from the ileal loop tests are reasonably consistent with the vascular permeability data, since those cultures which showed good activity in the one test also showed good activity in the other (see Tables 1 and 2).

Table 3 gives ileal loop data for six different toxigenic strains grown in syn/yeast for 24 hr at 37°. On the basis of this one test, it is impossible to state definitively that some cultures are more prolific producers of enterotoxin than others; but the data suggest that there may be a significant difference among the strains.

C. Selection of High Enterotoxin Producing Strains

In the third quarterly report it was stated that certain drug-resistant variants of strain 197 were isolated and found to produce at least 10-fold more enterotoxin than the parent strain.* Table 4 shows data from several experiments in which the amount of enterotoxin produced by two drug-resistant variants and the parent strain were compared. In each case, the drug-resistant organism was grown overnight in peptone (2%) broth with the drug, and these cultures were then used as the inoculum for either syn/yeast medium or syn/yeast medium plus the appropriate drug. The data show that when the inoculum (for syn/yeast) of the drug resistant variant is prepared in the presence of the drug and subsequently grown in the presence of the drug, at least 10-fold higher toxin production is obtained relative to the parent strain prepared and grown in the absence of the drug. Further, the data show that the drug-resistant variants do not produce significantly greater amounts of enterotoxin when they are grown (in syn/yeast) in

*Work done independently; patentability being explored.

the absence of the drug. One possible explanation of this result may be that the drug specifically induces "ent" plasmid replication. This possibility is presently under investigation.

In some cases, it was necessary to grow the drug-resistant variants for 48 hr instead of 24 hr in order to achieve increased toxin production. This was because the organisms grow more slowly in the presence of the drug. The decreased growth rate may account for the observation that a large difference in toxin production was not obtained in one experiment (test #2). Nevertheless, the data strongly suggest that there is a relationship between drug-resistance and toxigenicity, at least in the variants of strain 197 presently available. These observations are the basis for continuing experiments. The availability of a reliable high enterotoxin producing strain should aid significantly in the purification of the enterotoxin from E. coli strain 197.

II. Immunological Reagents for Detection of Enterotoxin and the Rabbit Skin Test for Vascular Permeability

In an earlier report (second quarterly), it was observed that hard, sometimes erythematous lumps developed at the site of inoculation several days after completion of a rabbit skin test for (E. coli toxin) vascular permeability. Sera obtained from such rabbits showed multiple immunoprecipitin lines when diffused against concentrates of E. coli culture filtrates. On the basis of this observation, some rabbits were held and immunized at various intervals (after completion of a skin test) with E. coli antigens (high speed sediments from ammonium sulfate concentrates, see Part III). The sera from one particular rabbit continued to show multiple immunoprecipitin lines against crude E. coli antigens. Testing of this antiserum (of which there is approximately 100 ml) to identify which of the antigen/antibody lines was toxin/antitoxin proved difficult because of the multiplicity of precipitin lines and the lack of a purified enterotoxin preparation. Also, the antiserum did not react with cholera toxin at the concentrations tested. When tested for its ability to neutralize a semi-purified preparation of E. coli enterotoxin in rabbit skin, however, some neutralization was observed (see Table 5). Because the dilution of antiserum which neutralized the toxin challenge (tested at 200 BD_{4mm}/ml) was low (1:8), the antiserum seems to be directed primarily against antigens other than the enterotoxin, most probably endotoxin(s). As such, this antiserum may not be useful in detection of enterotoxin, but rather in determining the presence of extraneous antigens in purified toxin preparations.

Concurrently with these experiments, two goats were immunized with coli antigens using a schedule and an adjuvant system previously employed successfully with cholera antigens. Both goats received semi-purified preparations of E. coli enterotoxin (see Part III) but one preparation contained less endotoxin than the other. The goat receiving the less-purified preparation succumbed during the experiment (apparently due to causes unrelated to

immunization). The second goat, however, survived and yielded an antiserum which precipitates and neutralizes both cholera and E. coli enterotoxin (Table 5). A sample of the antiserum (obtained 1 week before exsanguination) in fact, detects at least 5 μg/ml of cholera toxin by standard double immunodiffusion tests. The data in Table 5 suggests that approximately 200 BD_{4mm} of E. coli toxin is slightly greater than or equal to 1 Lb dose of cholera toxin (about 0.05 μg) as determined by neutralization with cholera antitoxin (Swiss Serum Vaccine Institute horse cholera antitoxin). Since 1 Lb dose of cholera toxin represents about 2000 BD_{4mm}, the data suggest a 10-fold difference in potency, at least with respect to vascular permeability activity. It is also noteworthy that the goat E. coli antitoxin sample neutralized 200 BD_{4mm} of coli toxin and 1 Lb dose of cholera toxin, respectively, to about the same extent (see Table 5).

None of these findings (as well as other findings in this report) could have been so easily obtained if it were not for the availability of the rabbit skin test. In our original research proposal, it was hypothesized that the rabbit skin test could be used to assay for E. coli enterotoxin, although evidence in the literature indicated otherwise (Sack et al, J. Infect. Dis., 123:378-385, 1971). Since the beginning of our studies we have consistently obtained evidence that the enterotoxin produced by different E. coli strains is capable of causing vascular permeability changes in rabbit skin which can be measured quantitatively on the basis blueing lesions, just as is the case with cholera toxin. (The recent publication of Evans, Evans and Gorbach, Infect. & Immun., 8:725-730, 1973, corroborates the observation that E. coli enterotoxins exhibit permeability factor activity.) So far, the test has centered on the determination of blueing doses BD_{4mm} -- wherein the endpoint is defined as that dilution of toxin which yields a 4 mm blueing lesion 18 hr after inoculation of 0.1 ml into rabbit skin. (The actual blueing dose value is given per ml -- i.e., 10X the dilution at which 0.1 ml yields a 4 mm lesion.) The 4 mm lesion was chosen as a suitable endpoint because titration of numerous enterotoxin samples showed that a linear relationship between blueing and dilution generally occurred only in the region of toxin dilution which yielded between 5 mm and 3 mm blueing lesions (Figure 1). Using the 4 mm endpoint, it has been possible to follow toxin recoveries during purification (see Part III). In addition, the evidence indicates that the observed blueing lesions (which are attributed to changes in vascular permeability) are due to heat-labile enterotoxin and that they are a measure of the same functionality detected by the rabbit ileal loop test. This is based on the facts that E. coli toxin samples heated to 100° for 2" lose 80% or more of their activity in both tests, E. coli toxin samples incubated with cholera antitoxin (SSVI antitoxin) are neutralized in both tests, and E. coli toxin samples with high levels of activity in the one test also exhibit high levels of activity in the other; while samples with low levels of activity exhibit low titers in both tests.

III. Purification of Enterotoxin

The first approach taken to concentrate E. coli enterotoxin from strain 197 culture filtrates was the metaphosphate precipitation technique employed for the concentration of cholera toxin. It was observed by rabbit skin

vascular permeability assays that the enterotoxin precipitated in the region of pH 4.0 to pH 4.5 (as does cholera toxin), but that recoveries were consistently poor (on the order of 10-20%). Follow-up studies indicated that the enterotoxin was labile at acid pH. Representative culture filtrates in the presence and in the absence of metaphosphate were adjusted to different pH's and subsequently (after 1 hr at room temperature) were adjusted back to either pH 7.0 or pH 8.5, the initial pH of the culture filtrate. Rabbit skin assays (Table 6) showed that culture filtrates lost significant vascular permeability activity in the absence of metaphosphate (2.55 g/l) when the pH was adjusted to pH 6.0 or below. In the presence of metaphosphate, the activity appeared to be less susceptible to acid pH, remaining essentially unchanged until the pH was lowered to below pH 5.0. Since the bulk of the toxin precipitated at or around pH 4.0, the poor recoveries alluded to above were attributed to the acid lability of the toxin. While these observations preclude the use of the metaphosphate technique for the concentration of toxin, acid-lability may be useful as a potential method for detoxification of enterotoxin or for conversion of the toxin into its subunit forms.

Also, there is the possibility that the lability of the toxin is concentration-dependent, as is the case with cholera toxin (J. P. Craig, personal communication). In that event, the metaphosphate precipitation technique might be more successfully employed using culture filtrates with increased levels of enterotoxin or at some later stage of purification.

As an alternative method of concentration, the enterotoxin from strain 197 was precipitated with ammonium sulfate (90% saturation). Reasonably reproducible recoveries of enterotoxin on the order of 50 to 100% were obtained in four out of six trials (Table 7). Using ammonium sulfate concentrates as the starting material for purification, it was discovered that the bulk of the enterotoxin activity could be significantly purified by repeated (twice) high speed sedimentation (78,140 $\times$ g, 16 hr, Spinco 30 rotor). Recovery data for a representative experiment are given in Table 8 and acrylamide gel patterns for the same samples described in Table 8 are given in Figure 2.

The results of standard acrylamide gel electrophoresis, together with the data in Table 8, indicated that the enterotoxin was amongst the group of proteins which migrate at or near the top of the separating gel (Figure 2). Subsequent experiments in which unfixed gel slices were eluted with buffer and then assayed in the rabbit skin test, in fact, indicated that the enterotoxin was located at the very top of the separating gel. Some activity was also detected at the top of the spacer gel, suggesting the existence of aggregates or some other form of the enterotoxin.

After sedimentation, enterotoxin preparations were dialyzed against high salt (2.5 M NaAc, pH 8.3) in an effort to dissociate aggregate forms (toxin/toxin or toxin/endotoxin complexes). Subsequent sedimentation of the enterotoxin from the high salt solution resulted in the separation of the

toxin and other high molecular weight (h.m.w.) contaminants presumed to include endotoxin (on the basis of Limulus and immunodiffusion assays) from the bulk of smaller contaminating proteins. The end result of these procedures was to obtain a purified preparation of enterotoxin, save for the presence of high molecular weight endotoxin(s). The problem then resided in the separation of the enterotoxin from the high molecular weight contaminants.

One approach to the problem was column chromatography. When a representative enterotoxin preparation (which had been sedimented from the high salt buffer) was chromatographed on Sepharose 4B (with an exclusion limit of about 15×10^6 daltons), two major peaks were recovered both of which contained enterotoxin (Figure 3). In this instance, the fractions were assayed by the change in morphology of adrenal Y1 (mouse) cells (Donta et al., Science 183:334-335, 1974). On the basis of mobility on Sepharose 4B, there appears to be a distribution of enterotoxin forms, some of which are associated with molecules of greater than or equal to 15×10^6 M.W.

Proceeding on the assumption that the enterotoxin in the void volume peak (Figure 3) was complexed in some way with endotoxin, high salt preparations of toxin were treated with urea (8 M) in an effort to dissociate the two moieties. Treatment with urea resulted in the disappearance of most of the opalescence usually associated with these preparations. Acrylamide gel electrophoresis revealed that the urea treatment resulted in the dissociation of the toxin into a small molecular weight "subunit" form (Figure 4), leaving the endotoxin apparently in its native form (with respect to size). SDS acrylamide gel electrophoresis indicated that the protein was on the order of 40,000 to 50,000 M.W. Since the toxin was primarily in a small molecular weight form, the endotoxin could be substantially removed by sedimentation. This procedure (with the exception that the urea concentration was reduced to 2 M) was used for preparing the antigen employed in raising the goat antitoxin previously described. On the basis of antigen neutralizing and precipitating properties of the goat serum, it would appear that treatment of the toxin with urea did not significantly alter its antigenicity.

In one experiment, treatment with urea followed by dialysis (to remove urea) and sedimentation (to remove endotoxin), resulted in the recovery of about 70% of the initial activity in the supernatant fraction and about 24% of the activity in the sediment fraction (Table 9). This result suggested that either the low molecular weight form of the toxin was biologically active or that it recombined upon removal of urea and endotoxin (by sedimentation) to form the native h.m.w. enterotoxin. Another possibility is that treatment with urea under the conditions employed did not quantitatively degrade all of the enterotoxin and that the activity observed in the supernatant fraction represents some native toxin (not completely removed by sedimentation).

In any event, these results suggest one possible solution to the problem of separating the h.m.w. toxin from its h.m.w. contaminants. By temporarily or permanently decreasing the size of the toxin (with urea or any other suitable reagent), a separation of it from persisting h.m.w. contaminants can be easily achieved either by sedimentation, chromatography, or selective ultrafiltration. If, after treatment with urea, the toxin remains primarily in its l.m.w. form, results from the goat immunization indicate that it may be an appropriate immunizing antigen. If, on the other hand, the toxin returns to its native form upon removal of urea, then the purification of the functional enterotoxin may be assured. All of these observations are the subject of continuing experiments.

Further, acrylamide gel electrophoretic patterns of crude enterotoxin preparations (see Figure 2, AMSC and 30K supernatant fractions) suggested the presence of a large amount of protein in a region of the gel occupied by the l.m.w. toxin form described above. This observation suggested that the toxin may exist as a h.m.w. and a l.m.w. form in representative culture filtrates. We are attempting to purify the material found in the filtrates to determine whether or not it represents a molecule identical to the urea-degradation product described above.

Finally, it has been determined that the E. coli enterotoxin is not physically identical to cholera toxin on the basis of electrophoretic mobility. Figure 5 shows the electrophoretic patterns of a semi-purified preparation of enterotoxin (AMSC, Sed #2 solution) in the presence and absence of cholera toxin. The gel with cholera toxin present shows that neither the toxin (heavy staining band) nor choleragenoid (faint band about 1/4" from the top of the gel interface) are electrophoretically identical with E. coli enterotoxin (presumed to be at the interface of the spacer and separating gels). Also, immunodiffusion of cholera toxin and E. coli enterotoxin against cholera antitoxin showed that the two antigens shared a common determinant, but were not identical (Figure 6). When both antigens were tested against coli (goat) antitoxin, however, they appeared to be immunologically indistinguishable (Figure 5). Further testing to confirm these findings are in progress.

TABLE I. Summary of Enterotoxin Production as determined by the Rabbit Skin Vascular Permeability Test

Strain & Lot No.	Medium	Time of Harvest (h)	Amount of Enterotoxin BD_4/ml Culture Filtrate
408-3 -1	Syncase	24	≤40 (1)*
408-3 -2	"	48	320 (1)
408-3 -3	"	24	<20 (1)
408-3 -4	"	24	25 (1)
408-3 -5	Syn/yeast	24	60 (1)
408-3 -6	"	24	82 (1)
408-3 -7	"	24	63 (1)
408-3 -8	"	24	<40 (1)
197 -1	Syn/yeast	24	226 (1)
197 -2	"	24	110 (3) (91-142)**
197 -3	"	24	283 (1)
197 -7	"	24	452 (4) (200-680)
197 -8	"	24	≥1500 (1)
197 -9	"	24	141 (1)
197 -10	"	24	269 (1)
197 -12	"	24	72 (1)
197 -14	"	48†	1313 (1)
197 -15A	"	24	381 (1)
197 -15B	"	48†	160 (1)
197 -17A	"	24	171 (1)
197 -17B	Syncase	24	<80 (1)
197 -18	Syn/yeast	24	184 (1)
197 -19B	"	24	149 (1)
197 -20	"	24	>1280 (1)

*Numbers in parentheses indicate number of separate determinations

**Two numbers in parentheses indicate range of values

†Culture incubated at 37° for 24 hr and held at 4° for 24 hr before harvesting.

Syn/yeast = Syncase medium supplemented with 0.6% yeast extract

TABLE 2. Summary of Enterotoxin Production as determined by the Rabbit Ileal Loop Test

Strain, Lot No.	Conc. (mg/ml) or Dilution Tested	Ileal Loop Test No.	Medium	Avg. Ratio (ml/cm)
408-3 -1P	1X culture fluid	2	Peptone	2.0 (4)*
408-3 -0	1X culture fluid	2	Syncase	1.2 (4)
Cholera Toxin	0.00036	2	--	1.45 (4)
408-3 -1P	1X culture fluid	3	Peptone	2.3 (4)
408-3, XM 50 conc. Ft. Det.	0.1	3	--	2.15 (4)
Cholera Toxin	0.00036	3	--	1.68 (4)
408-3 -1	1X culture fluid	6	Syncase	0 (4)
408-3 -2P	1X culture fluid	6	Peptone	0 (4)
408-3, XM-50 conc. Ft. Det.	0.25	6	--	1.4 (4)
Cholera Toxin	0.00725	6	--	1.07 (4)
408-3 -4	1X culture fluid	7	Syncase	0 (4)
408-3, XM-50 conc. Ft. Det.	0.25	7	--	0.70 (4)
197 -1	1X culture fluid	8	Syn/yeast	1.33 (4)
408-3 -8	1X culture fluid	8	Syn/yeast	0.29 (4)
Cholera Toxin	0.00725	8	--	1.69 (4)
197 -2	1X culture fluid	9	Syn/yeast	1.78 (4)
Cholera Toxin	0.00725	9	--	1.71 (4)
197 -17A	1X culture fluid	11	Syn/yeast	1.08 (4)
197 -17B	1X culture fluid	11	Syncase	0.54 (4)
Cholera Toxin	0.01	11	--	1.30 (4)
197 -19B	1X culture fluid	12	Syn/yeast	1.16 (4)
197 -19B	1:10	12	Syn/yeast	0.86 (4)
197 -20	1X culture fluid	12	Syn/yeast	1.14 (3)
197 -20	1:10	12	Syn/yeast	0.79 (3)
Cholera Toxin	0.001	12	--	1.07 (3)
"	0.0001	12	--	1.02 (3)

*Number in parentheses indicates number of loops measured in two rabbits (two loops per rabbit)

TABLE 3. Comparison of Toxin Production by Different Toxigenic *E. coli* Strains as Determined by The Rabbit Ileal Loop Test

Strain	Medium	Avg. Ratio (ml/cm)
106	Syn/yeast	0.9
334	Syn/yeast	1.06
197	Syn/yeast	1.33
408-3	Syn/yeast	0.29
10407	Syn/yeast	1.06
210	Syn/yeast	1.35
Cholera toxin 7.25 µg/ml		1.69

TABLE 4. Comparison of Production of Enterotoxin by Strain 197 and Selected Drug-Resistant Variants

Test No.	Strain	Inoculum Medium	Toxin Production Medium	Vol. of Culture (ml)	Time of Harvest (h)	BD_4 mm/ml
1	197	2% Peptone	Syn/yeast	80	24	360
	197-B ($\alpha^R\beta^R$)	2% Peptone + α(50)* β(250)	Syn/yeast	80	24	170
	197-B ($\alpha^R\beta^R$)	2% Peptone + α(50) β(250)	Syn/yeast + α(50), β(250)	80	24	[1500]**
2	197	2% Peptone	Syn/yeast	800	24	381
	197-B ($\alpha^R\beta^R$)	2% Peptone + α(50), β(250)	Syn/yeast + α(50), β(250)	800	24	625
3	197	2% Peptone	Syn/yeast	80	24	1000
	197-A(α^R)	2% Peptone + α(250)	Syn/yeast + α(250)	80	48	[7000]
4	197	2% Peptone	Syn/yeast	80	24 48 72	<640 [320] [320]
	197-A(α^R)	2% Peptone + α(250)	Syn/yeast	80	24 48 72	[320] [580] [200]
	197-A(α^R)	2% Peptone + α(250)	Syn/yeast + α(250)	80	24 48 72	<640 4850 [5280]
5	197	2% Peptone	Syn/yeast	1300	24	135
	197-A(α^R)	2% Peptone + α(250)	Syn/yeast + α(250)	1500	28	1930

*α = Antibiotic #1 resistance
β = Antibiotic #2 resistance

*Numbers in parenthesis indicate concentration of drug in μg/ml

**Numbers in brackets indicate extrapolated value.

TABLE 5. Neutralization Properties of Different Antisera

Serum	Dilution which yields a 4 mm blueing lesion after incubation with *E. coli* enterotoxin (200 BD_4/ml)	Dilution which yields a 4 mm blueing lesion after incubation with 1 Lb dose cholera toxin (0.05 μg/ml)
Rabbit #2 final bleed out	1:8 (test #1)	nt
SSVI, 2AU*	>2 AU (test #1)	≈1.5 AU (test #2)
Goat #2822	>1:256 (test #2)	>1:512 (test #2)
Sample bleeding 1 wk. before bleed out	≈1:400 (test #3)	≈1:480 (test #3)

*Swiss Serum + Vaccine Institute Standard (horse) cholera antitoxin. 2AU = 2 antitoxin units. One AU is defined as that amount of antitoxin which, when incubated with 1 Lb of cholera toxin, yields a 4 mm blueing lesion in rabbit skin.

TABLE 6. Effect of pH on Vascular Permeability Activity of *E. coli* Enterotoxin Strain 197 Culture Filtrates: In the Presence or Absence of Metaphosphate (2.55 g/l)

Strain, lot No.	pH	MP	Blueing Dose BD_4 mm/ml
197-7	8.5 → 7.0	-	577
	7.0 → 7.0	-	456
	6.0 → 7.0	-	394
	5.0 → 7.0	-	246
	4.0 → 7.0	-	149
	3.0 → 7.0	-	<16
197-7	8.5 → 8.5	-	≈325
	7.0 → 8.5	-	≈650
	6.0 → 8.5	-	<160
	5.0 → 8.5	-	<160
	4.0 → 8.5	-	<80
	3.0 → 8.5	-	≈45
197-9	8.5 → 8.5	+	150
	7.0 → 8.5	+	125
	6.0 → 8.5	+	200
	5.0 → 8.5	+	165
	4.0 → 8.5	+	<40
	3.0 → 8.5	+	<20

MP = Metaphosphate

TABLE 7. Recovery of Enterotoxin After Concentration by Ammonium Sulfate Precipitation as Determined by the Rabbit Skin Test

Sample	Volume (ml)	BD_4 mm/ml	Total BD_4 mm	Recovery (%)
197-10, IX culture filtrate	4630	269	1,245,000	100
AMS Concentrate	155	2985	462,675	37
AMS Supernatant	≥4630	<20	<92,600	<10
197-9, IX culture filtrate	5850	113	661,050	100
AMS Concentrate	290	3429	994,410	>100
AMS Supernatant	≥5850	nt	nt	nt
197-17A, IX culture filtrate	1000	30.3	30,300	100
AMS Concentrate	40	2690	107,600	>100
AMS Supernatant	≥1000	nt	nt	nt
197-14, IX culture filtrate	5300	1240	6,572,000	100
AMS Concentrate	390	4370	1,704,000	26
AMS Supernatant	≥5300	<20	<110,000	~2
197-18, IX culture filtrate	3300	69.6	229,800	100
AMS Concentrate	134	1902	254,900	>100
AMS Supernatant	≥3300	nt	nt	nt
197-19B, IX culture filtrate	1300	149	194,000	100
AMS Concentrate	60	1545	93,000	48
AMS Supernatant	≥1300	nt	nt	nt

TABLE 8. Recovery of E. coli Strain 197 Vascular Permeability Factor after Sedimentation (78,140xg, 16 h, Spinco 30 Rotor)

Sample	Volume (ml)	BD_4 mm/ml	Total BD_4 mm	Recovery (%)
197-9, AMSC	200	746.3	149,260	100
197-9, AMSC Sediment #1 solution	20	11,140	222,800	>100
197-9, AMSC Supernatant #1	20	174.1	3,482	2
197-9 AMSC Sediment #2 Solution	10	9,696	96,960	65
197-9, AMSC Supernatant #2	10	2,039	20,390	14

AMSC = Ammonium sulfate concentrate

TABLE 9. Effect of Urea on E. coli 197 Vascular Permeability Activity

Sample	Volume (ml)	BD_4 mm/ml	Total BD_4 mm	Recovery (%)
Lot 197-10 & 11 NAAC 30K Sediment solution	4.00	2000	8000	100
After 8 M urea, dialysis & sedimentation 30K supernatant	5.25	1055	5539	69
After 8 M urea, dialysis & sedimentation 30 K sediment	2.00	857.2	1914	24

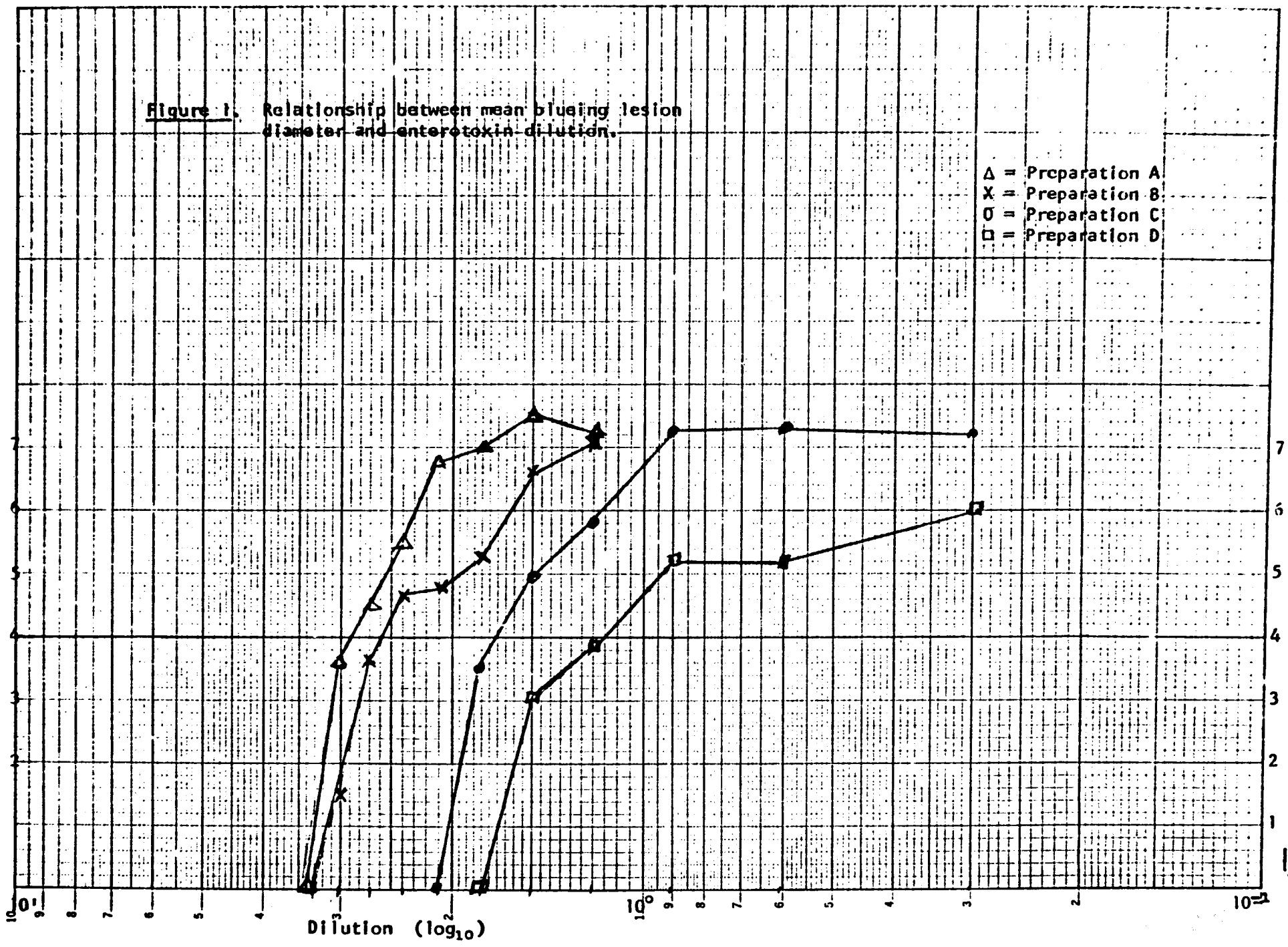

Figure 2. Standard acrylamide gel patterns of various E. coli (strain 197) fractions.

From left to right: AMSC (197-9); AMSC, 30 K Sup. #1 (197-9); AMSC, 30 K Sed. #1 (197-9); AMSC, 30 K Sup. #2 (197-9); and AMSC, Sed. #2 (197-9).

(AMSC = Ammonium sulfate concentrate)

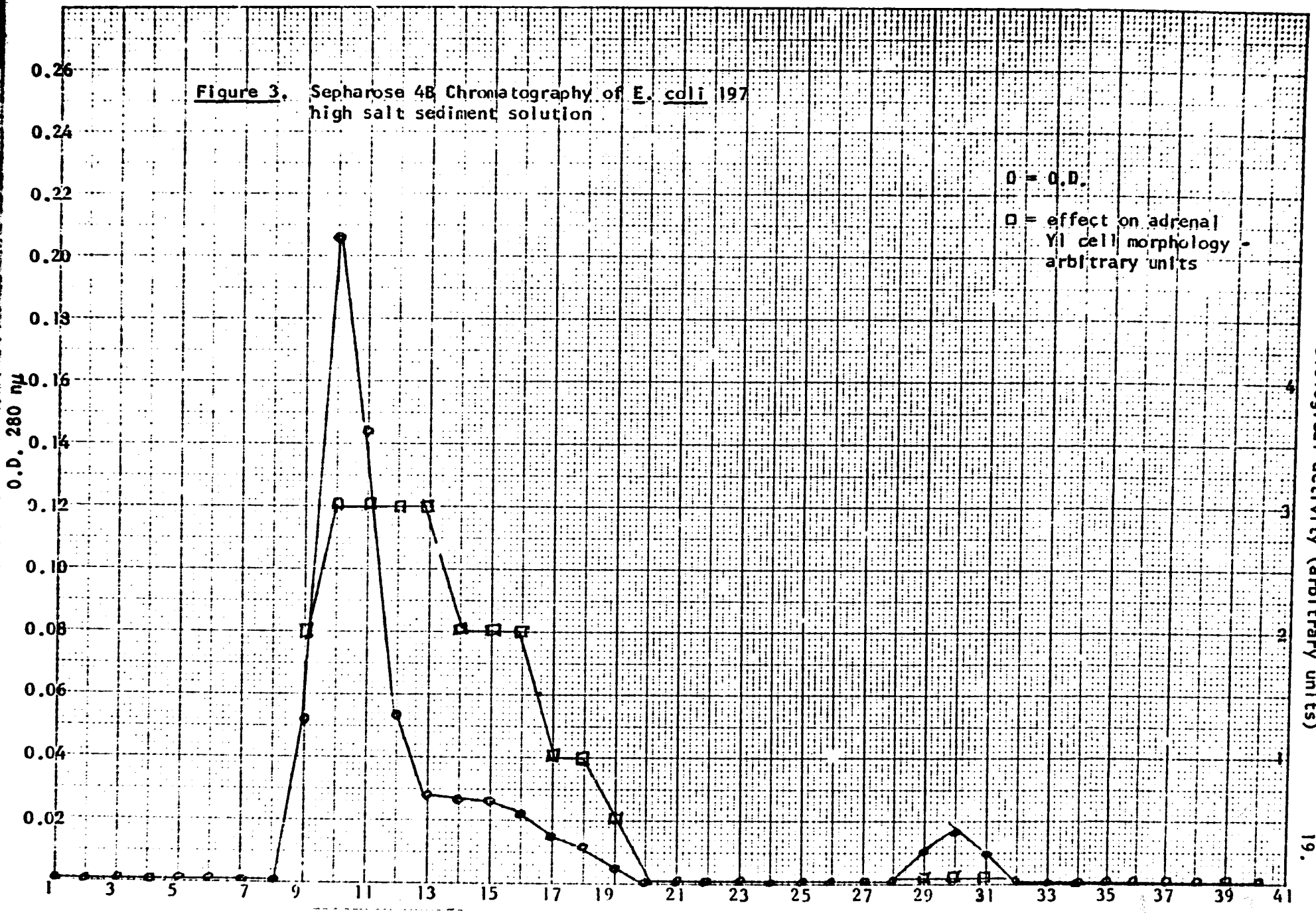

Figure 3. Sepharose 4B Chromatography of E. coli 197 high salt sediment solution.

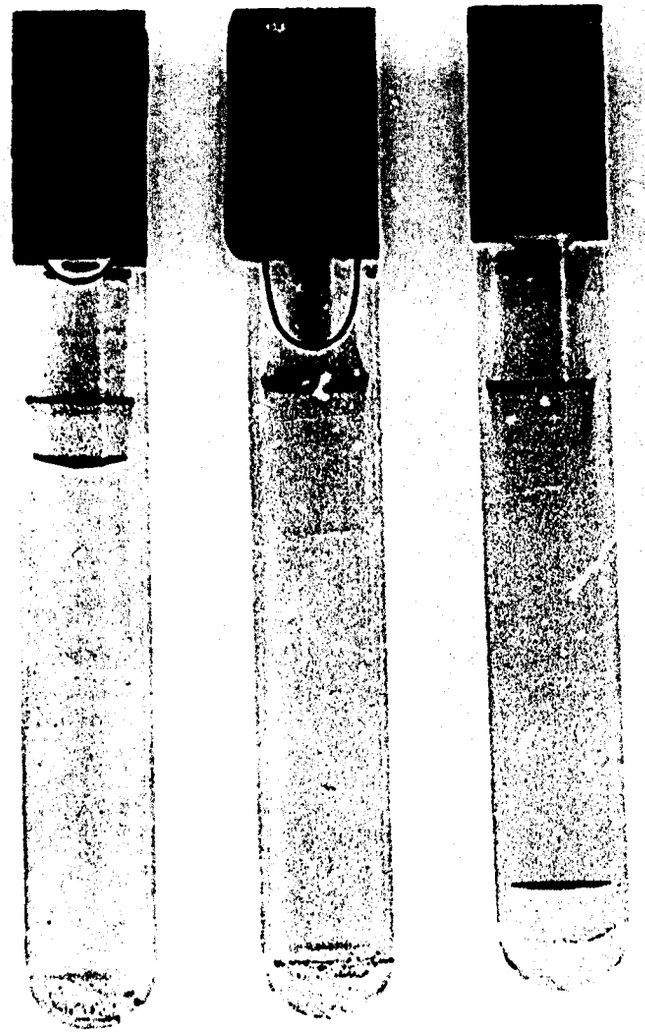

Figure 4. Standard acrylamide gel patterns of E. coli (strain 197) fractions after dialysis against high salt at pH 8.3 and after treatment with urea (series 197-10 and 11)

From left to right: 2.5 M NaAc 20 K sup.
2.5 M NaAc 20 K sed.
2.5 M NaAc 20 K sed. after treatment with 8 M urea

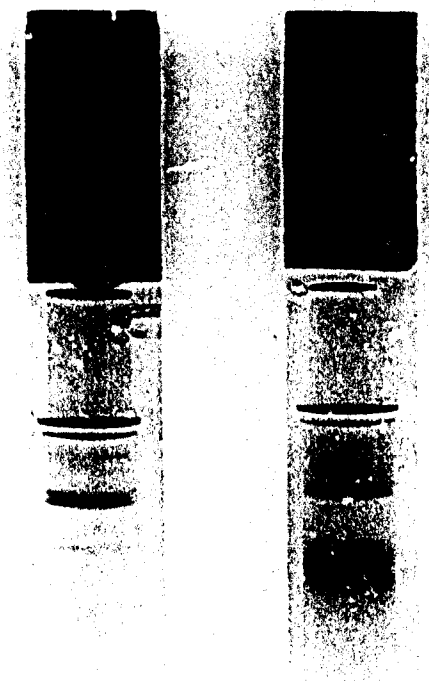

Figure 5. Standard acrylamide gel patterns of semi-purified E. coli enterotoxin (strain 197) with and without cholera enterotoxin.

From left to right: AMSC, Sed. #2;
AMSC, Sed. #2 + 50 µg cholera enterotoxin

(AmSC = Ammonium sulfate concentrates)

Figure 4. Double immunodiffusion of Cholera and E. coli toxins against cholera (goat) and E. coli (goat) antitoxin.

Wells 1 and 2 - Cholera goat #1 antitoxin
Wells 3 and 4 - Cholera toxin, 1.0 µg/ml
Wells 5 and 6 - E. coli goat #502 antitoxin
Center well - E. coli 107-19A ammonium sulfate concentrate