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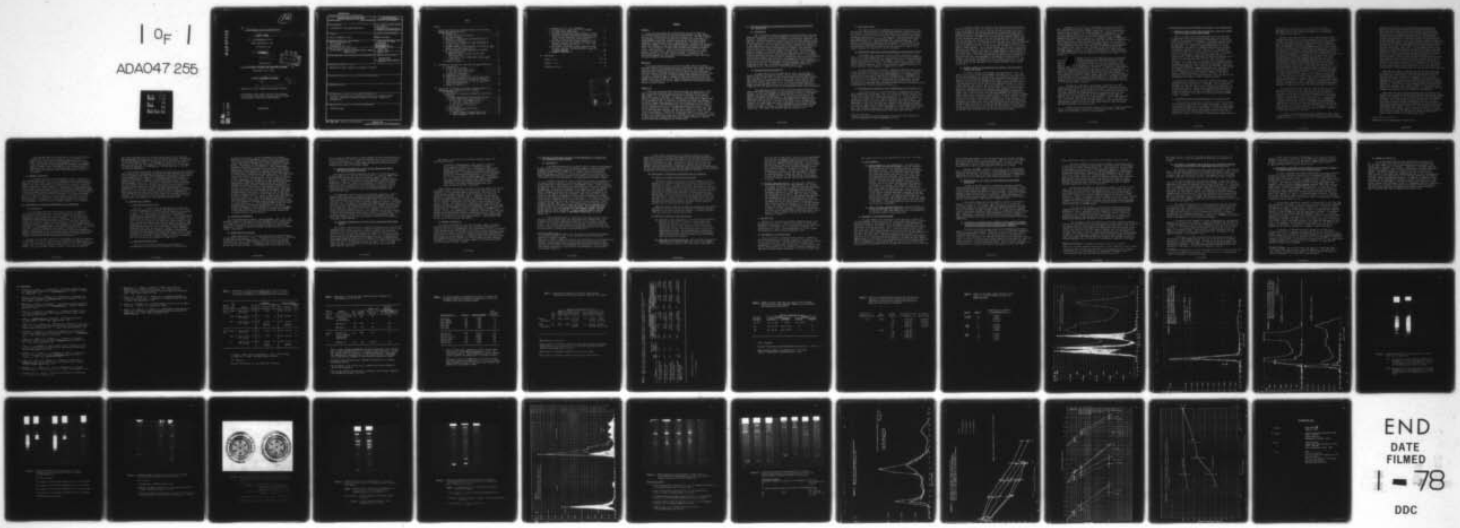

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SUMMARY

Section I

It has been determined that representative culture filtrates from two different strains (H197 and 74-114) of enterotoxigenic E. coli contain at least two different forms of heat-labile enterotoxin. One form of enterotoxin appears to be complex and partially insoluble; and on the basis of chromatographic properties, contains molecules of high molecular weight (ranging in size from about 2×10^5 to about 20×10^6). The other form of enterotoxin appears to be a single polypeptide chain of about 68,000 molecular weight and it shares at least one common antigenic determinant with cholera toxin. The distribution of these forms of enterotoxin depends on whether or not trypsin activable activity is taken into account. It is the complex, insoluble form of the enterotoxin which is activated by trypsin and the excision of a "protein" may be involved. The nature of the relationship between the forms as well as the mechanism of their activation by trypsin remains to be elucidated.

Section II

It has been determined that insol ECT stimulates rabbit circulating antitoxin specific for E. coli enterotoxin (soluble) and that trypsin-activated insol ECT is more antigenic than unactivated insol ECT. In contrast, it was determined that cholera (ga) toxoid, with or without adjuvant, stimulates antitoxin capable of neutralizing both cholera and E. coli enterotoxins. Most important, it was shown that a combined antigen formula consisting of both E. coli and cholera antigens is capable of stimulating antitoxin titers far greater than either antigen alone. These findings may have important implications in the design of a vaccine capable of providing long-lasting protection against diarrheal disease.

Section III

It has been established that the enterotoxigenic E. coli strain, 74-114, and its heat-labile extracellular products, soluble and insoluble ECT, are each capable of eliciting significant secretory responses in the ligated rabbit loop model for diarrhea. In addition, it has been shown that such secretory responses may be partially reduced when the samples are administered in the presence of LBTI, an inhibitor of trypsin-like proteolytic enzymes. The results suggest that in vivo proteolytic activation of at least one form of the enterotoxin (insoluble) may play a role in pathogenicity, although a conclusive demonstration of this has been hampered by the inability to control certain experimental variables. In addition, a comparative study of E. coli 74-114 and Inaba 569B indicates that the latter strain is at least 10^2 - 10^3 times more pathogenic. (Preliminary results with Ogawa 395, a more typical cholera vibrio pathogenic strain, is in agreement with this observation.) This finding is consistent with properties of various purified and partially-purified E. coli enterotoxin preparations which show a marked reduction in specific activity when they are compared with cholera toxin.

I. Partial purification and characterization of heat-labile Escherichia coli enterotoxins

A. Introduction

One of the major problems encountered in the purification of E. coli enterotoxins continues to be the heterogeneity associated with enterotoxic molecules. Depending on the culture conditions, purification techniques, and possibly, the strains employed, biologically active molecules ranging in size from about 20,000 to $\geq 1 \times 10^6$ in molecular weight, may be isolated from extracts of the organisms or from cell-free culture fluids. Although early reports (1-3) described properties of the high molecular weight forms of the enterotoxin ($\geq 10^6$ daltons), more recent investigations (4-7) appear to be concerned primarily with the low molecular weight forms (20,000 to 100,000 daltons), which are thought to be closely related to cholera toxin. The relationship between the different forms of heat-labile E. coli enterotoxin still remains to be elucidated as does their relationship to cholera toxin. In the studies reported here, emphasis has been placed on determining the relative distribution of the different enterotoxins present in representative culture filtrates, rather than on the complete and quantitative purification (and characterization) of one particular form. Nevertheless, the data presented reveal important properties of and clear cut differences between different enterotoxins elaborated by one or more strains of enterotoxigenic E. coli.

B. Strains and culture conditions

E. coli strain 74-114 (isolated from an infant with diarrhea in Brooklyn, New York and obtained from Dr. John P. Craig, Downstate Medical Center, Brooklyn, New York), a strain which produces only heat-labile enterotoxin (see Section III) was employed in most of the studies reported here. Some studies (described here and in the 1975 Annual Contract Report) were carried out using E. coli strain H197, a strain which produces both heat-stable and heat-labile enterotoxins, but this strain has been gradually replaced by 74-114 in order to eliminate the heat-stable factor as a consideration.

The organisms were grown at 37°C in the yeast extract-containing medium described by Evans et al. (8) with aeration provided by vigorous rotary shaking. Cultures were generally harvested at about 48 hours, a time when extracellular vascular permeability factor (PF) activity was found to be maximal (1975 Annual Contract Report). The organisms were removed by low speed centrifugation and the clarified supernatants were filtered through a Millipore filter (0.45 μm). The sterile cell-free culture filtrates were stored at 4°C, conditions under which no noticeable loss in PF activity was noted for at least 2 months. In some instances (to be described in another section), aliquots of culture filtrates were distributed into vials and lyophilized. The lyophilized materials were also stored at 4°C.

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C. Biological Assays

The rabbit skin vascular permeability factor (PF) assay (8, 9, 10) was used for routine analysis of samples before and after concentration and/or purification. Estimates of activity were based on the reciprocal of the last 2- (or 4-) fold dilution which elicited a 4 mm blueing lesion 18 hours after intradermal inoculation of 0.1 cc, i.e. BD_{4mm}/ml. In many instances, samples were analyzed both by the PF assay and by the Y-1 mouse adrenal cell assay described by Donta (11). When the adrenal cell assay was employed, the activity of enterotoxin preparations was estimated only on the basis of the morphological transformation (rounding) of the cells. Endpoints of activity were determined from the reciprocal of the last dilution (of sample) which caused rounding of at least 50% of the cell monolayer.

D. Methods for concentration and fractionation of enterotoxin molecules

Forty-eight hour, cell-free culture filtrates from either E. coli H197 or 74-114 were concentrated 10- to 40-fold by ultrafiltration, employing a Sartorius ultrafiltration unit and membrane filters with a 50,000 molecular weight cutoff (Science Essentials Co., Anaheim, California). Initial volumes usually ranged from 1 to 4 liters. With this method of concentration, no PF activity was ever observed in the filtrate fraction, whereas recoveries of PF in the retentate fraction generally ranged from 70 to 90%.

E. coli enterotoxin concentrates, prepared as described above, were used as the starting material for various attempts at purification. Depending on the experiment, such material was chromatographed at room temperature on a variety of columns such as Agarose A50-M, Sepharose 4B, or Sephadex G-150. Except where noted otherwise, the columns were equilibrated with 0.05 M tris HCl, pH 7.8-8.0, containing 0.02% sodium azide and the same buffer was used for elution of 7.5 ml fractions. The fractions were monitored for protein concentration by measuring their absorbance at 280 nm and for biological activity by assaying their PF and/or adrenal cell activity.

E. Distribution of enterotoxin forms elaborated by E. coli strain 74-114

After fractionation on (5x100 cm) columns of Sephadex G-150, enterotoxin concentrates from E. coli strain 74-114 were reproducibly resolved into three distinct peaks of biological activity (Figure 1). The first peak eluted in the void volume of the column (approximately 540 ml) followed closely by another active peak which was slightly retarded by the column (peak 2). The third peak of activity, which eluted in a volume of about 880 ml, was clearly separated from the first two peaks and it appeared as an ascending shoulder of a larger, inactive protein peak (see Figure 1). For purposes of discussion, the first two peaks of activity were designated insoluble[1] enterotoxin I (insol ECT I) and insoluble enterotoxin II (insol ECT II), respectively; while the third peak of activity was designated soluble enterotoxin (sol ECT).

[1]The term insoluble was employed because these materials were opalescent in physical appearance and were sedimentable at 10^5xg.

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In order to obtain an estimate of the relative distribution of the different enterotoxin forms, the fractions from each peak were pooled and the pools were assayed for PF activity, before and after treatment with trypsin. Trypsin treatment was employed because previous studies established that a significant portion of E. coli heat-labile enterotoxin was synthesized in a prototoxin form (10). The results of two separate chromatograms showed that sol ECT represented about 80% of the total recoverable activity before treatment with trypsin, but only about 20% of the total activity, after treatment with trypsin (Table 1). This was due to the fact that the PF activity associated with the insoluble forms was activated 15- to 100-fold after treatment with trypsin, whereas the activity of the soluble form increased only 2- to 5-fold after the same treatment. (It is unclear whether the low level of activation observed in the case of sol ECT was due to the presence of some contamination with (trailing) molecules from peak 2 or indeed represents true activation of the soluble form. Other data (to be described) suggest the former possibility to be more likely.) Thus, the data show that the distribution of enterotoxin forms recovered after chromatography is considerably altered depending on whether or not trypsin-activatable activity is taken into account. In view of these observations, the questions of the interrelationship of insol and sol ECT as well as the importance of the trypsin phenomenon take on added significance.

F. Further studies on the properties of soluble E. coli enterotoxin from E. coli 74-114

In order to examine in more detail the properties of sol ECT from strain 74-114, pools of the third Sephadex G-150 peak (Figure 1) were used as the starting material for further study. An estimate of the size of this form of the enterotoxin was obtained by chromatographing representative concentrates on a (2.5x100 cm) Sephadex G-75S column. In this instance, the fractions were monitored by the adrenal cell assay and the activity was found to be associated with a single major protein peak which eluted in a volume larger than the void volume of the column (Figure 2). The elution profile of activity indicated that sol ECT was less than 70,000 in molecular weight. Essentially identical results were obtained with preparations of sol ECT from strain H197 which had not first been chromatographed on Sephadex G-150. Rather, the sol ECT from strain H197 had been separated from high molecular weight enterotoxin by selective ultrafiltration, employing a Sartorius membrane filter with a cutoff of 160,000 molecular weight. In this instance, sol ECT was recovered in the filtrate fraction whereas insol ECT was recovered in the retentate. The adrenal cell activity associated with such filtrates (after concentration) eluted in the void volume of (2.5x100 cm) Bio-gel P-60 columns, but was slightly retained on (2.5x100 cm) Sephadex G-75S columns (Figure 3), as was sol ECT from strain 74-114 (Figure 2). On the basis of these results, it was tentatively concluded that sol ECT from both strains 74-114 and H197 was greater than 60,000 and less than 70,000 in molecular weight.

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Although sol ECT eluted in a single major peak from either Bio-gel P60 or Sephadex G-75S columns, analysis of the active materials by polyacrylamide gel electrophoresis (PAGE) revealed that sol ECT comigrated with numerous other proteins of similar size (Figure 4). Subsequently, it was found that batch treatment of sol ECT preparations (obtained from any of the above-mentioned columns or by selective ultrafiltration) with QAE-Sephadex A-25 resulted in the removal of a substantial number of extraneous proteins (Figure 5). When conditions were such that the anion exchanger and the toxin preparations were equilibrated with 0.05M tris-HCl, pH 7.3, many of the extraneous proteins adsorbed to the exchanger whereas sol ECT was found in the supernatant (Table 2). When the pH of the materials was adjusted to 8.3, however, most of the proteins including sol ECT were bound by the exchanger (data not shown). By selectively adsorbing out inactive protein with QAE-Sephadex A-25 at pH 7.3, then, it was possible to reduce the number of proteins contained in sol ECT preparations from about 20 to about 7 or 8 (Figure 5).

In order to determine which of the remaining proteins corresponded to sol ECT [illegible]liquots of QAE supernatants were electrophoresed and eluted from 2 m[illegible]es of unfixed acrylamide gels. With this approach, it was determi[illegible]t the major peak of adrenal cell activity eluted (from the gels) [illegible]position corresponding to the protein which exhibited the second greatest mobility on acrylamide gels (Figure 5). In addition, it was possible to obtain a more precise estimate of the size of sol ECT by examining the behavior of PAGE-purified toxin upon SDS acrylamide gel electrophoresis. Even after treatment with reducing agents and boiling, the toxin migrated as a single band with a mobility slightly greater than that of human serum albumin, a protein about 70,000 in molecular weight (Figure 6). The effect of trypsin on sol ECT was also investigated and preliminary results indicated that such treatment did not result in an alteration in either electrophoretic mobility or in PF activity (data not shown).

In addition to the above, immunological properties of sol ECT preparations were compared with those of cholera toxin in Ouchterlony-type double diffusion tests. In was found that sol ECT, irrespective of the state of purity, exhibited an immunoprecipitin line of identity with cholera toxin when these materials were diffused against E. coli (goat) antitoxin (the preparation of which was described in the 1974 Annual Contract Report). However, when the same samples were diffused against cholera (goat) antitoxin (12), only partial identity was observed (Figure 7). Thus, on the basis of this series of studies, sol ECT was identified as a single polypeptide chain, approximately 68,000 in molecular weight, which shares at least one common antigenic determinant with cholera toxin.

Ongoing studies are directed towards utilizing the information gathered herein for the purpose of isolating in a reproducible manner, significant quantities of purified sol ECT for further analysis.

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G. Further studies on the properties of insoluble E. coli enterotoxins

1. Summary of early studies with insol ECT from strain H197 (taken from 1975 Annual Contract Report (ACR))

Most of our early work on the characterization of heat-labile E. coli enterotoxins was performed using E. coli strain H197 and it was in this system that activation of enterotoxin by trypsin was first demonstrated (10). In contrast to the present studies, insol ECT from strain H197 was obtained in relatively pure form not by chromatography, but rather by repeated sedimentation (at 10^5xg) from Sartorius concentrates, a procedure which was also effective in achieving a separation of insol from sol ECT. Preparations of insol ECT isolated in this manner consisted, like insol ECT from strain 74-114 (Figure 1), of two classes of molecules. On the basis of their chromatographic properties (on Sepharose 4B and 6B, respectively), it was estimated that one class consisted of molecules $\geq 20 \times 10^6$ in molecular weight and the other class consisted of molecules on the order of 10^6 in molecular weight (Figures 6 and 7, 1975 ACR). The former class of molecules did not penetrate 3-1/2% acrylamide gels and the latter class did not penetrate 7-1/2% gels; and thus they appeared at the top and at the interface, respectively, of the spacer portion of standard acrylamide gels. Analysis of the two classes of insol ECT by SDS acrylamide gel electrophoresis further revealed that they were equally complex, consisting of about 18 identical components (Figure 5, 1975 ACR). Of the 18 components, only about five were predominant, the major one of which was estimated to be about 50,000 in molecular weight.

Working with a mixture of these two classes of molecules, it was demonstrated that treatment with trypsin resulted in a significant increase in PF activity (Table 17, 1975 ACR). This was the first evidence that insol ECT was the form of enterotoxin susceptible to activation by trypsin. Other studies suggested that treatment with trypsin resulted in an increase in the specific activity of both classes of enterotoxin and that the mechanism of activation might involve the excision (by trypsin) of a polypeptide chain (Figures 6 and 8, 1975 ACR).

2. Properties of insol enterotoxins from E. coli strain 74-114

In the studies reported here, properties of insol ECT molecules derived from E. coli strain 74-114 were investigated. Strain 74-114 was selected for study not only because it produced only heat-labile enterotoxin (as noted earlier) but because it produced significantly more prototoxin than did any other strain previously employed. A comparison of control and trypsin-treated culture filtrates from strains H197 and 74-114, for example, revealed that whereas elevations

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in PF activity on the order of 4- to 16-fold were generally observed with culture filtrates from strain H197, elevations on the order of 30- to >100-fold were obtained with like materials from strain 74-114 (Table 3).

Like insol ECT from strain H197, it is clear that insol ECT's elaborated by strain 74-114 are also activatable by trypsin (Table 1). In order to further study the trypsin effect, a pool of insol ECT I and insol ECT II was prepared by combining fractions from peaks 1 and 2 of a Sephadex G-150 column (Figure 1). The insol ECT's were then concentrated by repeated (3X) sedimentation at 10^5xg (16 hours at 4°C). The concentrate was divided into two equal parts: one part was treated with 100 mcg/ml trypsin (final concentration) for 2 hours at 37°C and the other part was incubated under the same conditions except with an equivalent volume (10% v/v) of 0.05M ammonium bicarbonate, the vehicle for trypsin. A comparison of the control and trypsin-treated ECT samples by PAGE revealed the appearance of two new protein bands in the trypsin-treated sample: one at the position occupied by the tracking dye (before staining) and the other at a position about one-fourth the distance into the lower gel (Figure 8). Previous studies with aliquots of the former band (eluted from acrylamide gels) showed that it was biologically inactive and did not form any precipitin lines when diffused against polyvalent E. coli antitoxin. One possible explanation for its increased intensity in the trypsin-treated sample is that it is a result of the comigration of trypsin (itself not detectable at the concentrations employed) with a weakly staining component present in control preparations. (Higher concentrations of trypsin, when electrophoresed under the same conditions, exhibit a band in the position of the tracking dye). However, the possibility that it represents a polypeptide (or glycoprotein) excised from insol ECT cannot be discounted and experiments designed to examine this possibility are contemplated (see below).

The second band of lesser mobility (which migrated to a position about one-fourth the distance into the lower gel) is apparently derived from insol ECT. As stated earlier, purified insol ECT preparations have generally exhibited two bands after PAGE: one at the top of the spacer gel (insol ECT I), and one at the interface between the spacer and lower gels (insol ECT II). In the present instance, several other proteins are present in the preparation (Figure 8) and they are thought to represent non-enterotoxigenic components derived from the organisms. Because the slower-moving band was also observed when more purified preparations of insol ECT were treated with trypsin (Figure 9), it is considered a likely possibility that this band is derived from insol ECT I, insol ECT II, or from both classes of molecules.

In order to further examine the trypsin phenomenon, control and trypsin-treated insol ECT (as depicted in Figure 8) were each chromatographed separately on a (2.5x45 cm) column of Sepharose 4B, equilibrated

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with 0.05 M tris, pH 7.8. The fractions from each column were monitored for protein concentration by measuring absorption at 280 nm and for biological activity by assaying for PF and adrenal cell activities. The elution profiles in both cases were similar to those previously obtained with (control and trypsin-treated) insol ECT from strain H197: biological activity was associated with a void volume peak (peak 1) and with a shoulder of optical density on the descending portion of the void volume peak (peak 2) (Figure 10). In both instances (control and trypsin-treated), the specific activity was greatest in fractions associated with the descending shoulder of the void volume peak. After treatment with trypsin, however, the specific activity (i.e. $BD_{4mm}/O.D._{280\ nm}$) associated with individual fractions in both peaks was about 15 times greater than in the same fractions obtained in the case of the control (untreated) material (Figure 10). These results suggested that both classes of molecules were activated by trypsin without significant change in their molecular weights. The fact that the void volume molecules exhibited lesser specific activity than the retained molecules may be due to the possibility that the former molecules represent aggregates or polymers of the latter molecules. This interpretation is consistent with the observation of a significant diminution in the opalescence of the trypsin-treated sample (relative to the control sample) and concomitantly, in the optical density of its void volume peak (Figure 10).

When the peak fraction (fraction #8) from the void volume peak of each column (control and trypsin-treated) was examined by SDS acrylamide gel electrophoresis, insol ECT was found to consist of the same components, irrespective of whether or not it had been treated with trypsin (Figure 11). Although there were numerous minor components (as in the case of insol ECT from strain H197), a major component of approximately 50,000 molecular weight was again observed (Figure 11). Just above this major component there was another component which migrated with a mobility similar to that exhibited by purified sol ECT from strain 74-114 (Figure 11). Whether or not this component corresponds to sol ECT remains to be determined.

When these and other fractions (obtained from the Sepharose 4B columns) were examined by regular PAGE, expected patterns were obtained (Figure 12): fractions in the void volume and its shoulder exhibited protein at the top and bottom of the 3-1/2% spacer gel, irrespective of whether or not they had been treated with trypsin. The only significant difference between the fractions from the two (control and trypsin-treated) columns was the presence (in fraction 18) of the trypsin-generated "protein" (Figure 12). This "protein", which did not exhibit either adrenal cell or PF activity (Figure 10), eluted in a volume less than that required for elution of trypsin[1] (fractions 21-23, Figures 10 and 12), and thus was identified as a molecule smaller than insol ECT II but larger than trypsin.

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[1]Determined from chromatograms of trypsin alone.

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Although PAGE permitted the detection of new "protein(s)" after treatment of insol ECT with trypsin, the small amounts of this material which were generated prevented extensive analysis. In order to define more precisely the mechanism of action of trypsin, either more material or new approaches are required. One approach which is presently under investigation is the preparation of C^{14}-labeled insol ECT, the use of which may permit a more reliable and more quantitative analysis of the origin and nature of the new "protein(s)".

H. Summary of Section I

It has been determined that representative culture filtrates from two different strains (H197 and 74-114) of enterotoxigenic E. coli contain at least two different forms of heat-labile enterotoxin. One form of enterotoxin appears to be complex and partially insoluble; and on the basis of chromatographic properties, contains molecules of high molecular weight (ranging in size from about 2×10^5 to about 20×10^7). The other form of enterotoxin appears to be a single polypeptide chain of about 68,000 molecular weight and it shares at least one common antigenic determinant with cholera toxin. The distribution of these forms of enterotoxin depends on whether or not trypsin activatable activity is taken into account. It is the complex, insoluble form of the enterotoxin which is activated by trypsin and the excision of a "protein" may be involved. The nature of the relationship between the forms as well as the mechanism of their activation by trypsin remains to be elucidated.

II. Antigenicity of insoluble, heat-labile E. coli enterotoxin

A. Introduction

It is well established that cholera toxin or toxoid are capable of stimulating protection-correlated circulating antitoxin in a number of experimental cholera models (13). It might therefore be expected that an appropriate E. coli antigen would behave similarly. Of the enterotoxins elaborated by enterotoxigenic E. coli, sol ECT appears to be more closely related to cholera toxin than insol ECT (Section I), and might therefore be the antigen of choice. However, the non-uniformity of sol ECT preparations prepared in different laboratories (4-7), together with the inability (thus far) to isolate it in reasonable quantity, present certain difficulties. In contrast, insol ECT, although biochemically more complex, is (because of its size) readily separated from the bulk of soluble proteins (and other contaminants present in representative culture filtrates); and it appears to exhibit similar physical and biological properties when isolated from two different enterotoxigenic strains (1975 Annual Contract Report and Section I).

Moreover, like sol ECT, insol ECT is also capable of producing a secretory response and this secretory response can be reduced when in vivo activation of insol ECT is blocked by trypsin inhibitor (Section III). These observations suggest that insol ECT may play as important a role as sol ECT in the pathogenesis of E. coli diarrheal disease. Further, it has been demonstrated

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that trypsin-activated culture filtrates require more antitoxin (and more G_{M_1} ganglioside) for neutralization than do unactivated culture filtrates (10). If the increased requirement for antitoxin is due to activation of insol ECT (as the data indicate), then it is reasonable to consider the possibility that insol ECT may itself be capable of stimulating antitoxin. On the basis of these considerations, an investigation of the antigenicity of insol ECT was therefore undertaken.

In the studies reported here, the ability of insol ECT to elicit circulating rabbit antitoxin was investigated both before and after treatment with trypsin. It was considered likely that trypsin-treated insol ECT might be more antigenic than untreated insol ECT because of the presumed unmasking (by trypsin) of antigenic determinants. In addition, the antigenicity of insol ECT was compared with that of a well-defined antitoxin-stimulating antigen, cholera (glutaraldehyde) toxoid (14). The data will show that 1) insol ECT isolated from two different strains is antigenic and elicits antitoxin specific for E. coli (soluble) enterotoxin; 2) trypsin-treated insol ECT antigen is approximately six times more antigenic than unactivated antigen; 3) cholera (ga) toxoid, with or without adjuvant, stimulates antitoxin which neutralizes both E. coli (soluble) and cholera enterotoxins; and 4) a combination of insol ECT antigen with cholera (ga) toxoid results in a synergistic antitoxin response, i.e., the combination of both antigens stimulates greater levels of antitoxin to both enterotoxins than does either antigen alone.

B. Preparation of antigens

1. Insol ECT from strain 74-114

Insol ECT I fractions from two Sephadex G-150 columns were pooled and concentrated by repeated (3X) sedimentation for 16 hours at 10^5x g. (Data pertaining to the starting materials are presented in Table 1.) A stock solution of insol ECT I was prepared by suspending the final sediments (see above) in 0.05 M tris-HCl, pH 7.8 containing 0.003 M EDTA and 0.02% sodium azide. Lowry determinations showed that the stock solution contained 1.2 mg/ml of protein. Just prior to use, an aliquot of the stock solution was divided into two parts: one part was treated with (sterile) trypsin (100 μg/ml, final concentration) for 1 hour at 37°C, and the other part was incubated under the same conditions with an equivalent volume of ammonium bicarbonate (10% v/v), the vehicle for trypsin. After incubation, (sterile) Lima Bean Trypsin Inhibitor was added to both samples (50 μg/ml final concentration) and they were each diluted to 60 μg/ml with sterile 0.067 M phosphate-buffered saline (PBS), pH 7.8, containing 0.01% thimerosal. Groups of rabbits were then immunized IM with 1 ml (60 μg) of each preparation, respectively.

2. Insol ECT from strain H197

Insol ECT was isolated from Sartorius concentrates of representative culture filtrates by repeated (2X) sedimentation

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(at 10^5x g for 16 hours). A pool of sediments from several production lots was suspended in 0.05 M tris-HCl, pH 7.8 containing 2 M urea and was then purified further by chromatography on a (5x100 cm) column of Agarose A-50 M, equilibrated with the same buffer. (The addition of urea resulted in a diminution of the opalesence associated with the material.) After chromatography (Figure 13), fractions contained in the major peak of PF activity (which behave on acrylamide gels like insol ECT II) were pooled, dialyzed extensively against 0.05 M ammonium bicarbonate, pH 8.3 (to remove urea), and the material was lyophilized. The lyophilized product was stored at 4°C until used. When dissolved in 0.05 M tris-HCl, pH 7.8 at a concentration of 10 mg/ml, the insol ECT was milky in appearance. The opalescence (or milky appearance) was significantly reduced by the addition of EDTA (0.005 M) and sodium desoxycholate (0.35% final concentration) and the opalescence remained diminished even after extensive dialysis against 0.02 M tris-HCl, pH 7.8. After dialysis, the material was diluted with the same tris buffer to a Lowry protein concentration of 500 μg/ml and thimerosal (0.01% final concentration) was added. This stock preparation was stored at 4°C. Just prior to use, aliquots were diluted to 50 μg/ml with sterile 0.067 M PBS, pH 7.8, which also contained thimerosal (0.01%). Groups of rabbits were then immunized with 50 μg doses (1 ml). When the antigen was administered in combination with cholera toxoid, the antigens were diluted so that the final preparation contained 50 μg of E. coli antigen and 100 μg of cholera toxoid in 1 ml. Since no significant PF activity could be demonstrated either before the addition of or removal of solubilizing agents, or after treatment with trypsin, the E. coli antigen was considered to be detoxified. (Lyophilization appears to inactivate the PF activity of insol ECT and may therefore be a suitable method for detoxification.)

C. Immunization parameters

Male, New Zealand albino rabbits, weighing between 7 and 12 lbs., were employed. Samples of pre-immune sera were obtained by heart puncture within 1 to 2 weeks prior to immunization. Groups of rabbits (8 per group) were immunized intramuscularly in the right posterior thigh at 0 and 6 weeks, respectively. Samples of sera were obtained, by heart puncture, at 6 and at 8 weeks, respectively.

D. Determination of antitoxin

Rabbit serum antitoxin titers were determined by the anti-PF assay as described by Craig et al. (15). The sera were inactivated for 30 minutes at 56°C and stored until used at -20°C. Two-fold dilutions of sera were incubated (for 1 hour at 37°C) separately with one limit-of-blueing (Lb) dose of cholera toxin and with 1 Lb dose of sol ECT (from strain 74-114). Sol ECT was derived from peak 3 of Sephadex G-150 columns (Figure 1) and

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its Lb content was determined by titration against the Swiss Serum and Vaccine Institute (SSVI) provisional standard cholera antitoxin (containing 4470 antitoxin units (AU) per ml). In each assay, the toxin challenges (cholera and sol ECT) were titrated against SSVI serum and the data obtained were used to standardize the results of different assays.

E. Comparative antigenicity of insol E. coli enterotoxins before and after activation by trypsin.

This study was designed to determine the effect of trypsin activation on the antigenicity of insol ECT from strain 74-114. Groups of rabbits were immunized with each antigen (control and trypsin-treated -- Section II, part B. 1.), respectively, according to the immunization schedule outlined in Section II, part C. The results of serum antitoxin titrations (Table 4) showed that 1) insol ECT, after two inoculations, is capable of eliciting significant levels of rabbit circulating antitoxin; 2) insol ECT elicits antitoxin which is highly specific for (sol) E. coli enterotoxin; and 3) trypsin-activated insol ECT is at least six times more antigenic than unactivated insol ECT.

The observation that two doses of insol ECT were required in order to achieve significant levels of circulating antitoxin suggests that the rabbit immune response (and perhaps the human response) may be similar to the immune response elicited by cholera (ga) toxoid. (Two doses of cholera toxoid are also required in order to achieve significant levels of circulating antitoxin (14)). In addition, the magnitude of ECT-directed antitoxin titers elicited by control and trypsin-activated insol ECT, respectively, were within the range of cholera antitoxin values elicited by cholera toxoid without and with adjuvant, respectively (13). Thus, treatment of insol ECT with trypsin may be an effective methodology for increasing the antigenic potency of (insol) ECT preparations (by as much as 6-fold). This observation, while of basic interest to those who are interested in understanding E. coli enterotoxin immunology, may also have economic implications for production of E. coli antigen vaccines.

F. Comparative antigenicity of insol E. coli enterotoxin and cholera toxoid

The purpose of this study was to examine the antigenicity of an insol ECT preparation from strain H197 and to compare its antigenicity with that of well-defined antigen, cholera (ga) toxoid. Groups of rabbits were therefore immunized with: 1) an insol ECT antigen from strain H197 (Section II, part B. 2.); 2) a cholera (ga) toxoid antigen (lot 20301); 3) the cholera toxoid administered with protamine/aluminum adjuvant (14); and 4) a combination of the E. coli antigen and cholera toxoid. Rabbits receiving preparations #2 and #3 were immunized approximately 2 months prior to rabbits receiving preparations #1 and #4. In each case, however, the rabbits were of the same approximate age, were purchased from the same supplier, and were housed under identical conditions.

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The results of serum antitoxin titrations (Table 5) permit the following conclusions:

> 1) antitoxin raised against E. coli antigen is specific for E. coli (sol) enterotoxin; 2) antitoxin raised against cholera toxoid is just as effective in neutralizing E. coli (sol) enterotoxin as it is in neutralizing homologous enterotoxin; 3) increased antitoxin elicited by cholera toxoid plus protamine/aluminum adjuvant is just as effective (if not more so) in neutralizing E. coli (sol) enterotoxin as it is in neutralizing cholera toxin; and 4) a combination of E. coli and cholera antigens elicits significantly greater antitoxin titers against both types of enterotoxin than is elicited by either antigen alone, both after primary and booster inoculations.

These findings indicate that insol ECT is not only capable of eliciting significant levels of circulating antitoxin (in the rabbit) but that its antigenicity is not affected either by the method of isolation or by the strain employed (compare antitoxin levels elicited by insol ECT from strains 74-114 and H197 in Tables 4 and 5). Moreover, the observation that antitoxin raised by insol ECT from two different strains (H197 and 74-114) was equally effective in neutralizing sol ECT from only one strain (74-114) supports the idea that insol ECT may not vary antigenically from strain to strain. The finding that a combination of E. coli and cholera antigens stimulates a synergistic antitoxin response to both toxins (E. coli and cholera) was unexpected in view of the specificity of E. coli antitoxin (Tables 4 and 5). It is possible that E. coli endotoxin (presumed to be present in the E. coli preparations) is involved. Alternatively, the E. coli antigen may possess a common antigenic determinant which is masked in the cholera toxoid and which, by itself (as in the case of the E. coli antigen alone), is incapable of eliciting cholera antitoxin. These possibilities are the topics of continuing investigation.

G. Summary of Section II

It has been determined that insol ECT stimulates rabbit circulating antitoxin specific for E. coli enterotoxin (soluble) and that trypsin-activated insol ECT is more antigenic than unactivated insol ECT. In contrast, it was determined that cholera (ga) toxoid, with or without adjuvant, stimulates antitoxin capable of neutralizing both cholera and E. coli enterotoxins. Most important, it was shown that a combined antigen formula consisting of both E. coli and cholera antigens is capable of stimulating antitoxin titers far greater than either antigen alone. These findings may have important implications in the design of a vaccine capable of providing long-lasting protection against diarrheal disease.

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III. The role of proteolytic activation in the pathogenicity of Escherichia coli-mediated diarrheal disease

A. Introduction

It was demonstrated earlier that the PF activity found in representative E. coli culture filtrates could be activated by (rabbit) intestinal proteolytic enzymes both in vitro and in vivo and that activation could be blocked by Lima Bean Trypsin Inhibitor (LBTI) (10). Further, when PF activation was prevented in vivo, a reduction in the secretory response also occurred (10). These findings suggested that proteolytic activation of enterotoxin might play an important role in the pathogenesis of E. coli-mediated diarrheal disease.

In order to examine the role of proteolytic activation in pathogenicity, the rabbit secretory response to various E. coli enterotoxin preparations was investigated both in the presence and in the absence of LBTI. In early studies, E. coli strain H197, which produces both heat-stable and heat-labile enterotoxins, was employed. Using culture filtrates from this strain, it was not possible to demonstrate a significant difference in the rabbit secretory response to samples incubated with and without LBTI unless the time of incubation of the fluids (in the ligated loops) was shortened. (This involved several intraluminal (in the same loop segment) inoculations and withdrawals, a procedure which was both tedious and traumatic for the animals.) The reasons for employing short-term incubations (up to 30 minutes) were: 1) to prevent inactivation of the inhibitor (LBTI) which was thought to occur during the normal 18 hour incubation period[1]; and 2) to limit the exposure time of pre-existing PF with intestinal mucosa[2]. Even when a reduction in the secretory response could be demonstrated (under conditions described above), the factors responsible for the residual secretory response which was observed (30-35% of control values) were not easily identified. It was considered feasible that the partial response was due either to heat-stable enterotoxin, or to pre-existing PF.

It has now been possible to obviate the short-term incubation method entirely, since conditions have been found under which standard ileal loop procedures can be used to demonstrate inhibition of the secretory response by LBTI. The problem of heat-stable enterotoxin was eliminated by employing strain 74-114, a strain which produces only heat-labile enterotoxin (Table 6) and the problem of pre-existing PF was eliminated by titrating (in ileal loops) a dilution series of enterotoxin with and without LBTI[3].

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[1] It was considered feasible that either the inhibitor was eventually digested by proteolytic enzymes or that it was eventually overcome by continued secretion of mucosal proteases.

[2] This was, in effect, a means of "diluting out" the contribution of pre-existing PF.

[3] When dilutions of culture filtrates from strain H197 were titrated in early studies, no significant secretory response was demonstrated even with small dilutions (2- or 4-fold). The difference in the potency of culture filtrates from strains H197 and 74-114 may be due to the difference in their content of prototoxin (Table 3).

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In the studies reported here, the secretory response to various preparations of enterotoxin was investigated in the presence and absence of LBTI. The preparations included both crude (culture filtrates) and partially purified enterotoxin (insol ECT I, insol ECT II, and sol ECT) as well as viable enterotoxigenic E. coli. The data will confirm that the secretory response (to enterotoxin) can be significantly reduced under certain conditions (when the contributions of both heat-stable enterotoxin and pre-existing PF are eliminated) and will support the idea that the effect may be due primarily to prevention of proteolytic activation (by LBTI) of insol ECT.

B. Description of enterotoxin preparations and micro-organisms

1. Crude E. coli enterotoxin (culture filtrate)

The sterile culture filtrates from a single production lot of strain 74-114 (prepared as described in Section I, part B.) was used throughout the study. In order to eliminate the effects of long-term liquid storage, aliquots (10 and 25 ml, respectively) of culture filtrate were lyophilized and then stored at 4°C. Just prior to use, each vial was rehydrated to its original volume with sterile distilled water and then diluted (when required) in enterotoxin production medium (EPM). When the effect of LBTI (on the secretory response) was investigated, various dilutions of the sample were divided into two equal parts: a given amount of LBTI was added to one part, and the other part served as a control. In some experiments, a single dilution of sample was employed and the amount of LBTI (added to multiple tubes of the dilution) was varied.

When the effect of heat on the secretory response was investigated, only undiluted culture filtrate was employed. The sample was again divided into two parts: one part was boiled for 15 minutes and the other part served as a control (not boiled).

2. Partially purified E. coli enterotoxins

a. Insol ECT I from strain 74-114. The insol ECT I preparation employed in this study was identical to the preparation described in Section II, part B. 1., except that it was diluted to a concentration of about 90 μg/ml with sterile 0.067 M PBS, pH 7.4, containing 0.1% gelatin. This stock solution was stored at 4°C throughout the study which lasted approximately 4 weeks. For each experiment, a sample was removed from the stock solution and divided into two equal parts: a given amount of LBTI was added to one part and the other part served as a control.

b. Insol ECT II from strain 74-114. The insol ECT II preparation employed in this study was obtained from the same Sephadex G-150 columns as was insol ECT I (see Figure 1 and Table 1).

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As in the case of insol ECT I, insol ECT II was concentrated from the pool of Sephadex fractions by repeated (2X) sedimentation and the sediments were dissolved in 0.05 M tris-HCl, pH 7.8, containing 0.02% sodium azide. For the studies reported here, a stock solution containing 30 μg/ml of insol ECT II was prepared from the opalescent sediment solution by diluting an aliquot with sterile 0.067 M PBS, pH 7.4, containing 0.1% gelatin. The stock solution was stored at 4° throughout the duration of the study (about 4 weeks). For each experiment, a sample was removed from the stock solution and divided into two equal parts: a given amount of LBTI was added to one part and the other part served as a control.

c. Sol ECT from strain 74-114. Like insol ECT I and ECT II, sol ECT was derived from the same Sephadex G-150 columns (see Figure 1 and Table 1). Fractions from the third peak of Sephadex columns were pooled and concentrated about 6-fold by dialysis against Ficoll (Pharmacia Fine Chemicals, Piscataway, New Jersey). After concentration, the sample was centrifuged for 16 hours at 10^5 x g in order to remove any contaminating insol ECT II. (No sediment was observed.) The supernatant was then filtered aseptically through a 0.45 μm filter and the protein concentration of an aliquot was determined (by the Lowry method) to be 600 μg/ml. A stock solution containing 200 μg/ml of sol ECT was prepared, using 0.067 M PBS, pH 7.4, containing 0.1% gelatin and the solution was stored at 4°C for the duration of the study (4 weeks). For each experiment, a sample was removed from the stock solution and divided into two equal parts: a given amount of LBTI was added to one part and the other part served as a control.

3. Cholera toxin

Cholera toxin, purified as described by Rappaport et al. (12), was employed as a control in many of the studies. Prior to each experiment, an aliquot of a stock solution containing 500 μg/ml was diluted with 0.067 M PBS, pH 7.4, containing 0.1% gelatin to a concentration of 5 μg/ml. The cholera toxin control (at 5 μg/ml) was tested both in the presence and absence of LBTI.

4. Heat-stable E. coli enterotoxin

For use as controls in assays for the presence or absence of heat-stable enterotoxin (in culture filtrates from strain 74-114), sterile culture filtrates from heat-stable only strains were produced as described in Section I, part B. The strains employed were designated E. coli 18B and B44, respectively, and they were obtained from Dr. Ralph Giannella, V. A. Hospital, Lexington, Kentucky. In each assay for heat stable enterotoxin, the culture filtrates (from

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each strain) were tested with and without boiling (for 15 minutes).

5. Micro-organisms

a. Enterotoxigenic E. coli strain 74-114. The growth from several fresh overnight (2% peptone) slants was harvested and suspended in a solution containing dextran, sucrose, and sodium glutamate. The suspension was then distributed into vials (0.2 cc/vial) and the vials were lyophilized. The lyophilized material was stored at -20°C until used. On the day before an experiment, from one to three vials were rehydrated each with one ml of 2% peptone and the contents from each vial were dispensed into 10 ml of 2% peptone. The cultures were allowed to incubate at 37°C for about 18 hours at which time they were centrifuged at 10,000 RPM for 10 minutes. The sedimented organisms from each culture were resuspended in 15-20 ml of EPM and re-centrifuged under the same conditions. After the second centrifugation, the supernatants were filtered through 0.45 μm filters and the organisms were again suspended in the same volume of EPM. Bacterial counts of the organisms were made. Before each experiment, the organisms were divided into several equal aliquots and varying amounts of LBTI were added to different aliquots. One aliquot, without LBTI, was saved as the control. The samples were kept in an ice bath during the course of the experiment.

b. Vibrio cholerae, serotype Inaba 569B. Exactly the same procedures as described for E. coli strain 74-114 were employed in the case of Inaba 569B, except that only one concentration of LBTI was tested in a given experiment.

C. Parameters of the ileal loop assay

The adult rabbit ileal loop assay (16) was employed to measure the secretory response to various (whole cell or cell-free) preparations of enterotoxin administered in the presence and in the absence of LBTI (Worthington Corporation, Freehold, New Jersey). Some assays were performed to test the heat-stability of the enterotoxin. Male, New Zealand albino rabbits, weighing about 2 kg, were deprived of food, but not water, for 48 hours prior to use. In a given test, 14-18 ligated segments were constructed along the length of the small intestines starting about 60 cm from the pylorus and ending about 30 cm from the ileal cecal valve. The average segment length was about 9 cm. All samples (1 ml in volume) were tested in duplicate: one sample in the upper half and one sample in the lower half of the length of intestines employed. When a series of dilutions of a sample were tested with and without a constant amount of LBTI, paired dilutions were injected side by side, but the position of the paired samples was randomized both in the upper and lower half of the intestines.

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This procedure was adapted in order to take into account variable protease levels in different regions of the intestines. When the dilution (of sample) was constant and the LBTI concentration was varied, the series of samples was randomized in both the upper and lower halves of the intestines. The concentrations of LBTI employed ranged from 0.04 mg/ml to 5 mg/ml.

Eighteen hours after incubation of the loops in the closed abdomen, animals were sacrificed by injection of sodium pentobarbitol, and the small intestines were removed. The volume of fluid contained in each segment was determined, and the length of the empty segment was measured. The secretory response per loop was expressed as the volume-to-length ratio (ml/cm).

D. Evidence that E. coli strain 74-114 produces only heat-stable enterotoxin

In order to determine whether the secretory response elicited by enterotoxins derived from E. coli strain 74-114 could be attributed, in part, to heat stable enterotoxin, samples of culture filtrate were assayed (in the ileal loop test) before and after boiling (15 minutes). Since the secretory response to heat stable enterotoxin is maximal at about 6 hours (17), animals were sacrificed at 6 and 18 hours, respectively. Known heat-stable only culture filtrates were employed as controls and they were also tested before and after heat treatment. EPM served as a negative control.

The results showed that boiling completely abolished the secretory response to culture filtrate from E. coli strain 74-114 at 6 hours and at 18 hours (Table 6). In contrast, the 6 hour secretory response elicited by two heat-stable only culture filtrates was reduced (relative to controls) only by 15-28% after the same treatment (Table 6). These results suggested that the heat-stable enterotoxin strains either produced small amounts of heat-labile enterotoxin or that heat-stable enterotoxin is partially sensitive to heat. Nevertheless, the results confirmed that the secretory response to enterotoxins derived from strain 74-114 could not be attributed to heat-stable enterotoxin.

E. The effect of Lima Bean Trypsin Inhibitor on the secretory response to heat-labile enterotoxin from strain 74-114: Comparison of various dilutions of culture filtrate with and without inhibitor

Culture filtrate from E. coli strain 74-114 (prepared as described in Section III, B. 1.) was tested for its ability to elicit a secretory response in the presence and absence of LBTI. Undiluted culture filtrate and a series of dilutions ranging from 1:2.5 to 1:25 were each tested in the presence of inhibitor, the concentration of which was varied (in each dilution) from 0.04 mg/ml to 5 mg/ml. In this series of studies, samples without inhibitor (undiluted and diluted culture filtrate) served as positive controls and EPM, with and without inhibitor, served as negative controls. Only the highest concentrations of inhibitor (1 or 5 mg/ml) were tested in the negative controls and under

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these conditions, no secretory response was ever observed (data not shown).

The results showed that the secretory response to undiluted culture filtrate was not significantly reduced when it was administered in the presence of from 0.04 to 5 mg/ml of LBTI (Figure 14)[1]. This observation was attributed to the presence of pre-existing PF (approximately 80 BD_{4mm}/ml). However, as the pre-existing PF was diluted out, a significant reduction in the secretory response was observed and it appeared to be dose dependent up to LBTI concentrations of 1 mg/ml (i.e., 5 mg/ml) did not further reduce the secretory response, which indicated that the proteolytic enzyme content of each loop did not exceed 2.5 mg/ml[2]. Nevertheless, the data were consistent with the hypothesis that the reduced secretory response was due to inhibition of intestinal proteolytic enzymes (by LBTI) required for activation of prototoxin.

As noted earlier (Table 3), the prototoxin content of culture filtrates from strain 74-114 was greater than observed in culture filtrates from other strains and this may account for the greater potency of the present material. Even though the PF titer of the undiluted culture filtrate was only 80 BD_{4mm}/ml, it was still capable of eliciting a secretory response at dilutions of 1:10 (Figure 14). This is in contrast to earlier studies in which even lower dilutions of culture filtrates with higher PF titers, but lower prototoxin content, were incapable of eliciting secretory responses. Thus, the actual potency (vis a vis secretory response) of E. coli culture filtrates may depend more on the prototoxin content than on pre-existing PF.

The fact that the secretory response was not completely abolished suggested that part of the response was not elicited by protease-activatable enterotoxin. This is consistent with the presence in culture filtrates of a soluble form of the enterotoxin (Section I, part F), which is either not activated or only slightly activated by trypsin (Table 1). Further, it is possible that part of the residual secretory response may be stimulated by prototoxin activated after the effect of the inhibitor is overcome[3].

Still another possible explanation for the residual secretory response involves the distribution of proteolytic enzymes along the length of the intestines. For example, when the data presented in Figure 14 were analyzed in terms of the response elicited by a given sample (with and without LBTI) in the upper region of the intestines versus the lower region, different dose response curves were obtained: the range of inhibitor concentrations employed was more effective in the upper region than in the lower region (some representative data are presented in Table 7). This obversation suggested that the protease content of the lower region was greater and therefore that more inhibitor was required (in order to achieve comparable levels of reduction in the secretory response in both upper

[1] Each point in Figure 14 represents the mean of from 10 to 44 loops.

[2] One mg of the LBTI preparation employed in this study inhibited 2.5 mg of trypsin.

[3] Preliminary data indicate that the inhibitor is effective for only a limited period of time. It is possible that it is eventually degraded or that it is overcome by additional proteases secreted by mucosal cells.

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and lower regions). Experiments designed to determine the distribution of proteolytic activity along the length of the intestines are presently in progress.

F. The effect of Lima Bean Trypsin Inhibitor on the secretory response to partially purified E. coli enterotoxin from strain 74-114.

The secretory response to partially purified insol ECT I, insol ECT II, and sol ECT were each investigated in the presence and absence of LBTI (1 mg/ml). In this series of experiments, positive controls consisted of cholera toxin (5 μg/ml), with and without LBTI (1 mg/ml), and negative controls consisted of 0.067 M PBS with 0.1% gelatin (pH 7.4), with and without LBTI (1 mg/ml)[1].

The results showed that the secretory responses to insol ECT I and insol ECT II were only partially reduced in the presence of LBTI, and that the magnitude of the reduction decreased slightly with time (Figure 15). Since the insol ECT preparations contained neither heat stable enterotoxin nor pre-existing PF[2], it was expected that a more dramatic reduction in the secretory response would occur (in the presence of LBTI). This expectation was based on the hypothesis that inhibition of trypsin-like intestinal proteases by LBTI would prevent proteolytic activation of insol ECT (considered to be prototoxin) and that this would result in a substantial reduction in at least the early (3 hr and 6 hr) secretory responses. Instead, the secretory responses elicited in the presence of LBTI ranged from about 58% (at 3 hours) to about 80% (at 18 hrs) of control values (Figure 15).

One possible explanation for these results may be that the test dose of enterotoxin was too large (insol ECT I was tested at 90 μg and insol ECT II was tested at 30 μg). Alternatively, prototoxin may have been activated by proteases which were either not exposed to or not susceptible to LBTI. Preliminary data (not shown), in fact, indicates that prototoxin is bound by G_{M1} ganglioside in *in vitro* experiments. If this occurs *in vivo*, then it is possible that prototoxin, bound by mucosal receptors, is subsequently activated either by extracellular or by intracellular proteases.

In contrast to the findings with insol ECT preparations, the secretory responses to cholera toxin, tested at 5 μg, were unaffected by LBTI (Figure 15). Although the magnitude of the secretory responses elicited by cholera toxin (at 6 hr and at 18 hr) was similar to those elicited by insol ECT I (at 6 hr and at 18 hr), it is plausible that the test dose of cholera toxin was also too great to be affected by LBTI. The possibility exists, therefore, that the secretory response to lesser amounts of cholera toxin (for example, 10 ng) might be affected in the presence of LBTI.

In the case of sol ECT, the reduction in the secretory response (in the presence of LBTI) ranged from 69% (at 3 hr) to 96% (at 18 hr) of control values (i.e., in the absence of LBTI). Whether or not the early reduction in the secretory response is significant will require further experimental (and statistical) evaluation. The slight reduction observed might be due to the presence of trace amounts of insol ECT II (Section I, part E).

[1] No secretory response was elicited by the negative controls, with or without LBTI.

[2] The insol ECT preparations exhibited little or no PF prior to treatment with trypsin or exposure to intestinal enzymes.

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Finally, the time course of development of the secretory response appeared to be similar irrespective of the enterotoxin preparation or of the presence of LBTI (Figure 15). This observation suggests that the mechanism of adenyl cyclase activation may be the same for the different forms of E. coli enterotoxin and for cholera toxin, as well.

G. The effect of Lima Bean Trypsin Inhibitor on the secretory response to enterotoxigenic E. coli and to Vibrio cholerae

In order to determine the possible significance of in vivo proteolytic activation of insol enterotoxin (prototoxin) for pathogenicity, enterotoxic strains were tested for their ability to elicit a secretory response in the presence and in the absence of LBTI. E. coli strain 74-114 was tested at cell concentrations of 2-4 $x10^8$ with varying amounts of LBTI, ranging from 0.04 mg/ml to 5 mg/ml. The secretory responses elicited by the E. coli samples were compared to those elicited by Vibrio cholerae, serotype Inaba 569B, which was tested at cell concentrations between $2x10^5$ and $2x10^6$. In the case of Inaba 569B, however, only the two highest concentrations of LBTI were employed (1 mg/ml and 5 mg/ml, respectively). In both cases (E. coli and Inaba 569B), the organisms tested without LBTI served as positive controls and the final wash fluids (Section III, part B. 5.) served as negative controls. The wash fluids were selected for negative controls in order to rule out the presence of extracellular enterotoxin in the inoculum; and in no instance was a secretory response elicited by these controls.

Since erratic results were obtained in the upper half of the intestines, only the secretory responses in the lower half of the intestines were evaluated[1] (see Section III, part C for the design of experiments). The results, which are expressed in terms of percent control values (i.e., the secretory response elicited by the organisms in the absence of LBTI), revealed that the secretory response to enterotoxigenic E. coli was significantly reduced in the presence of LBTI and that the reduction was dose-dependent (Figure 16). In contrast, the secretory response to 2-3 logs less of cholera vibrios was not significantly affected at the highest concentrations of LBTI (Figure 16). The reduction in the secretory response to E. coli 74-114 could not be attributed to a bacteriacidal effect of LBTI since these organisms, like Inaba 569B and Ogawa 395, grew well in the presence of varying amounts of LBTI (Table 8). The effect could, however, be attributed to a reduction of enterotoxin production in the presence of LBTI and this possibility is presently under investigation.

Irrespective of whether the results are due to an inhibition of proteolytic activation of E. coli prototoxin by LBTI (in the intestines) or alternatively, due to a reduction in enterotoxin production, the results, nevertheless, suggest that proteolytic activation (whether in the organisms themselves or in the intestines) may play an important role in the pathogenicity of enterotoxigenic E. coli strains. A consideration of the possible utility of appropriate protease inhibitors as prophylactics in the treatment of E. coli-mediated diarrheal disease may be indicated.

[1] In many instances, the positive controls (E. coli 74-114 and Inaba 569B, each without LBTI) did not elicit secretory responses in the upper half of the intestines, whereas the same samples in the same rabbits elicited full positive responses in the lower half of the intestines.

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H. Summary of Section III

It has been established that the enterotoxigenic *E. coli* strain, 74-114, and its heat-labile extracellular products, soluble and insoluble ECT, are each capable of eliciting significant secretory responses in the ligated rabbit loop model for diarrhea. In addition, it has been shown that such secretory responses may be partially reduced when the samples are administered in the presence of LBTI, an inhibitor of trypsin-like proteolytic enzymes. The results suggest that *in vivo* proteolytic activation of at least one form of the enterotoxin (insoluble) may play a role in pathogenicity, although a conclusive demonstration of this has been hampered by the inability to control certain experimental variables. In addition, a comparative study of *E. coli* 74-114 and Inaba 569B indicates that the latter strain is at least 10^2-10^3 times more pathogenic. (Preliminary results with Ogawa 395, a more typical cholera vibrio pathogenic strain, is in agreement with this observation.) This finding is consistent with properties of various purified and partially purified *E. coli* enterotoxin preparations which show a marked reduction in specific activity when they are compared with cholera toxin.

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TABLE 1. Distribution of heat-labile E. coli enterotoxins (from strain 74-114) after Sephadex G-150 chromatography: effect of trypsin activation on recoveries of soluble and insoluble enterotoxins.

Strain (Lot #)	Form of ECT	Sample	Vol. (ml)	UNTREATED PF[2]	UNTREATED Total PF	UNTREATED Recov.[3] (%)	TRYPSIN-TREATED[1] PF	TRYPSIN-TREATED[1] Total PF	TRYPSIN-TREATED[1] Recov.[3] (%)
74-114 (3,4,5)	Insol I	Seph. G-150; Fr. 70-90	145	40	5,800	6	≥2560	≥371,200	≥45
	Insol II	Seph. G-150; Fr. 95-110	116	171	19,836	20	≥2560	≥296,960	≥36
	Sol	Seph. G-150; Fr. 120-145	185	408	75,480	75	847	156,695	≤19
					101,116			≥824,855	
74-114 (8)	Insol I	Seph. G-150; Fr. 70-85	112	<40	<4,480	<10	3618	405,216	47
	Insol II	Seph. G-150; Fr. 90-100	88	26.4	2,323	5	2939	258,632	30
	Sol	Seph. G-150; Fr. 110-140	189	204	38,556	85	1076	203,364	23
					45,359			867,212	

[1].100 μg/ml trypsin (final concentration), 1 hour, 37°C, followed by addition of 50 μg/ml Lima Bean Trypsin Inhibitor

[2].PF = BD_{4mm}/ml

[3].Relative distribution of total enterotoxin recovered

TABLE 2. Recoveries of soluble E. coli enterotoxin after treatment with QAE-Sephadex A-25 at pH 7.3[1]

Strain (Lot #)	Stage of Purification	Vol. (ml)	Protein[2] (μg/ml)	Adrenal cell[3] activity units (per ml)	Specific Activity $\left(\frac{\text{Units of activity}}{\mu\text{g protein}}\right)$
197H (75,76L)[4]	Sartorius SM11730 filtrate (<160,000 M.W.)	120	5700	10^6	175
	QAE Sup #1	120	1200	10^6	833
	QAE Sup #2	120	734	10^6	1362
74-114 (2)	Bio-gel P60 (pool of peak #1 concentrated from 40 ml)	12.7	2900	10^5	35
	QAE Sup. #1	12.0	153	2.6×10^4	170

[1] Equal volumes of sample and QAE-A25 each equilibrated with 0.05 M tris-HCl, pH 7.3, were mixed and shaken for 15 minutes at room temperature. The QAE was removed either by settling or low speed sedimentation. The process was repeated until the yellow cast of the starting material disappeared. Final concentration of QAE-A25 in each extraction was about 80 mg/ml.

[2] Determined spectrophotometrically (Methods in Enzymology III, Colowick and Kaplan, pg 453)

[3] The reciprocal of the last dilution of sample which caused rounding of $\geq$50% of the monolayers.

[4] This lot was induced with (250 μg/ml) lincomycin (Levner et al., Abstracts 1976 ASM Meeting, Atlantic City, N.J.)

TABLE 3. Prototoxin content of representative culture filtrates from E. coli strains H197 and H74-114: comparison of PF activity in control and trypsin-treated samples

Strain (Lot #)	Control[1]	Trypsin-treated[2]	Fold difference in PF
H197 (56)	113	≥1280	>11
H197 (58+60)	76	387	5
H197 (61+62)	394	1575	4
H197 (63)	160	618	4
H197 (67)	92	1470	16
H197 (77)	69	1040	15
H74-114 (1)	299	15,510	52
H74-114 (2)	75	6,299	84
H74-114 (3,4,5)	46	12,600	274
H74-114 (3A)	63	4,303	68
H74-114 (6)	149	4,445	29
H74-114 (8)	178	11,360	64

[1]One ml aliquots were incubated at 37°C for 30-60 minutes with 50 μl of 0.05 M ammonium bicarbonate, pH 8.3, the vehicle for trypsin. After incubation, 25 μg of lima bean trypsin inhibitor was added per ml of culture filtrate.

[2]Fifty μg (in 50 μl) was added per ml of culture filtrate and the solution was incubated at 37° C from 30-60 minutes. After incubation, 25 μg of lima bean trypsin inhibitor was added per ml of culture filtrate.

TABLE 4. Comparative Antigenicity of Insol E. coli Antigen[1] from Strain 74-114 before and after Treatment with Trypsin

	Dose	MEAN ANTITOXIN RESPONSE (AU/ml)					
		Cholera Toxin Challenge			E. coli Toxin Challenge[2]		
		Weeks post-immunization			Weeks post-immunization		
	μg	0	6	8	0	6	8
Before activation	60	<2(7)	<2.0(7)	4.6(7)[3] (<2-16)[4]	nt	8.4(7) (<2-58)	473(7) (11-5203)
After activation	60	<2(8)	1.2(8)	9.1(8) (<2-69)	nt	28.1(8) (<2-139)	3016(8) (801-35,735)

[1] See Section II, part B. 1.

[2] The E. coli toxin challenge employed in this experiment was a partially purified preparation of soluble E. coli enterotoxin (68,000 molecular weight) from E. coli strain 74-114.

[3] The number in parentheses indicates number of sera tested

[4] The numbers in parentheses indicate the range of values observed

TABLE 5. Rabbit antitoxin responses to: 1) E. coli enterotoxin; 2) cholera (ga) toxoid; 3) cholera (ga) toxoid plus protamine/aluminum adjuvant; and 4) a combination of (1) and (2).

Antigen	Dose (μg)	Immunization Schedule (weeks)	MEAN ANTITOXIN RESPONSE (AU/ml) Cholera toxin challenge Weeks post-immunization 0	5-1/2 -6	8	E. coli toxin challenge[2] Weeks post-immunization 0	5-1/2 -6	8
1) insol E. coli antigen from strain H197[1]	50	0, 6	<2(8)	<2 (8)	3 (6)[3] (<2-266)[4]	nt	6 (8) (<2-37)	532(6) (15-1663)
2) cholera (ga) toxoid Lot 20301, plain	100	0, 6	<2(8)	28 (8) (8-52)	1860 (7) (1025-4710)	<2(8)	52 (8) (20-163)	5442(7) (495-154,140)
3) cholera (ga) toxoid Lot 20301 + protamine/ aluminum adjuvant	100	0, 6	<2(8)	172 (6) (97-543)	6155 (6) (1975-18842)	<2(8)	264 (6) (128-662)	19,280(6) (7982-70,420)
4) insol E. coli antigen plus cholera (ga) toxoid Lot 20301	50) 100)	0, 6	<2(8)	442 (7) (127-1264)	39,230(6) (8698-89,905)	nt	475 (7) (88-1241)	25,630(6) (11370-129,762)

[1] See Section II, part B. 2.

[2,3,4] - See Table 4

TABLE 6. Rabbit secretory responses to E. coli culture filtrates before and after heat treatment: comparison of heat-stable and heat-labile enterotoxins.

Culture filtrates	Secretory Response (ml/cm)			
	6 Hours		18 Hours	
	Control	Treated[1]	Control	Treated[1]
74-114[2]	.73± .11 (8)[3]	0 (8)	1.8± .06 (6)	0
B44	.32± .05 (8)	.23± .04 (8)	nt	nt
18B	.33± .05 (8)	.28± .05 (8)	nt	nt

[1] 100° 15 minutes

[2] Culture filtrate was lyophilized before testing (Sec. II, part B. 1.)

[3] Mean secretory response ± standard error of the mean. Number in parentheses indicates number of loops.

TABLE 7. Effect of Lima Bean Trypsin Inhibitor on the secretory response to culture filtrate from E. coli 74-114 as a function of loop location: comparison of responses in upper and lower halves of intestines.

Dilution of Culture filtrate	Loop location	I Conc. (mg/ml)	Secretory Response (ml/cm)	AV. (Upper + lower loops)
1:2.5	upper	0	1.26 (13)	1.46 (26)
		.04	1.16 (6)	1.38 (12)
		.2	0.80 (7)	1.13 (14)
		1.0	0.65 (8)	.78 (16)
		5.0	0.20 (4)	.57 (8)
	lower	0	1.66 (13)	
		.04	1.60 (6)	
		.2	1.46 (7)	
		1.0	0.90 (8)	
		5.0	0.94 (4)	

TABLE 8. Effect of Lima Bean Trypsin Inhibitor on the viability of enterotoxigenic *E. coli* and *Vibrio cholerae*

Strain	I (mg/ml)	Concentration of organisms (colonies per ml)
E. coli 74-114	0	1.55×10^9
	0.2	1.65×10^9
	1	2.07×10^9
	5	2.14×10^9
Inaba 569B	0	5.67×10^7
	0.2	5.70×10^7
	1	5.30×10^7
	5	5.70×10^7
Ogawa 395	0	9.3×10^7
	0.2	9.1×10^7
	1	1.0×10^8
	5	1.0×10^8

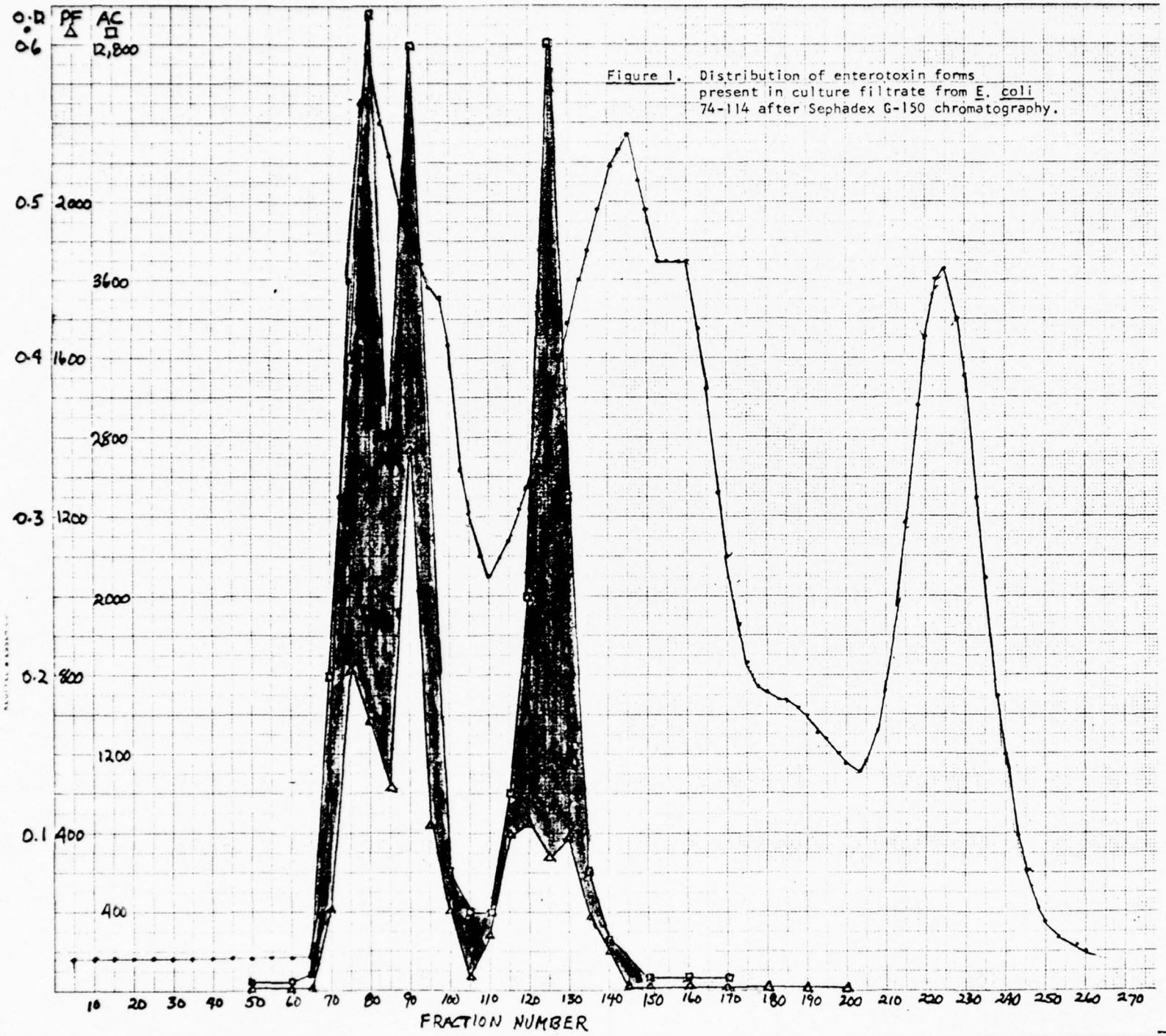


Figure 1. Distribution of enterotoxin forms present in culture filtrate from E. coli 74-114 after Sephadex G-150 chromatography.

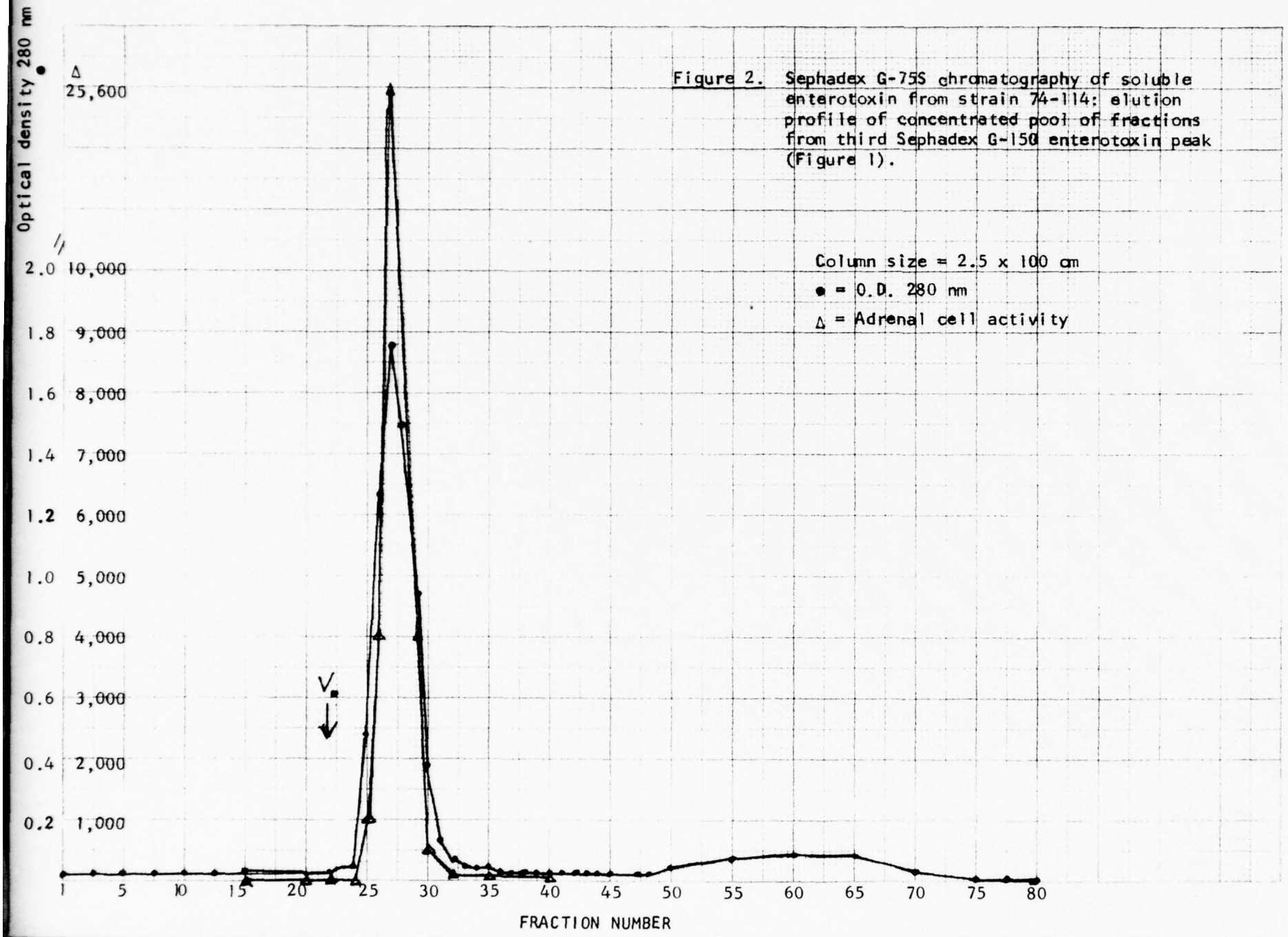


Figure 2. Sephadex G-75S chromatography of soluble enterotoxin from strain 74-114: elution profile of concentrated pool of fractions from third Sephadex G-150 enterotoxin peak (Figure 1).

Column size = 2.5 x 100 cm

● = O.D. 280 nm

Δ = Adrenal cell activity

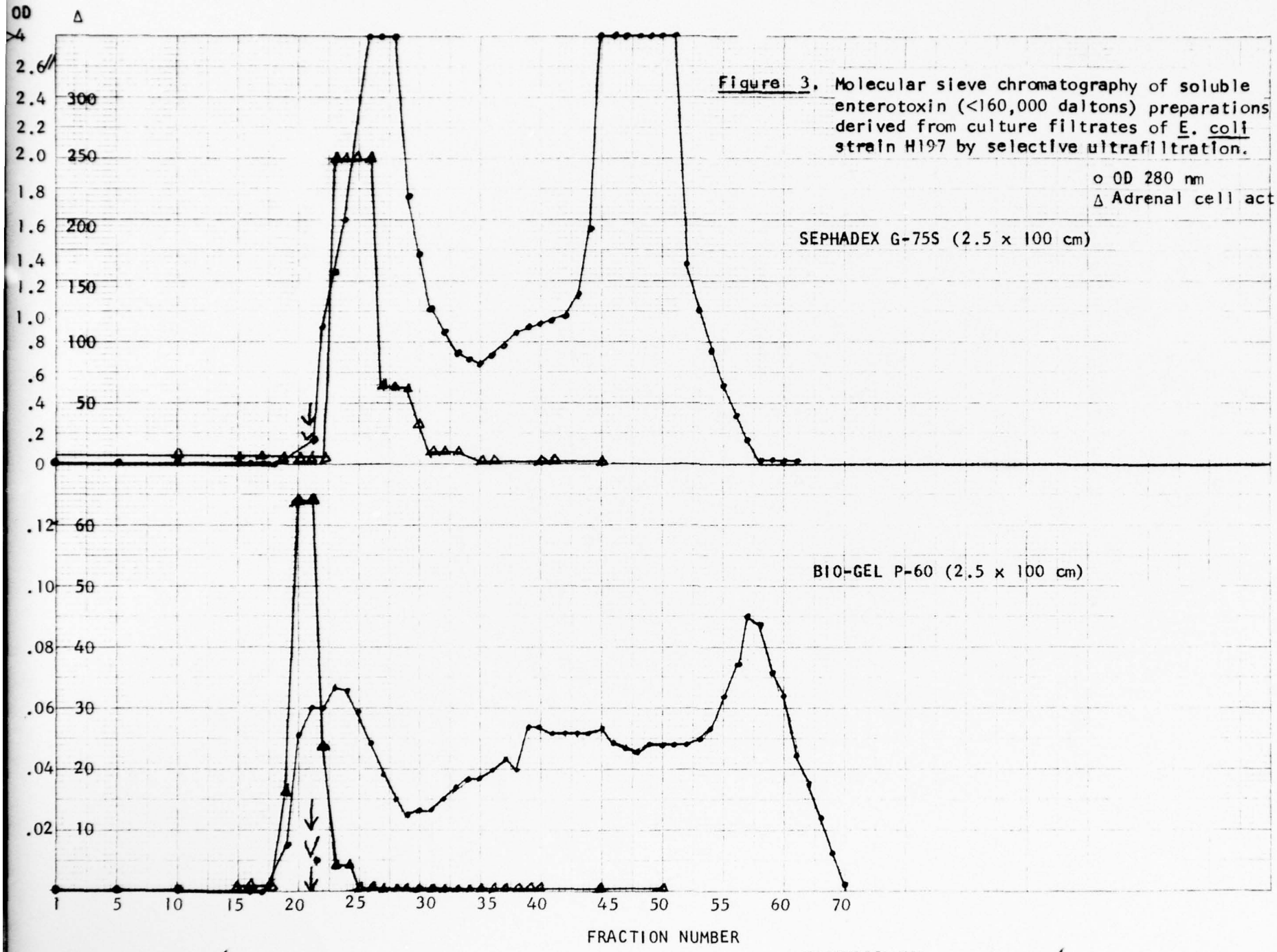


Figure 3. Molecular sieve chromatography of soluble enterotoxin (<160,000 daltons) preparations derived from culture filtrates of E. coli strain H197 by selective ultrafiltration.

o OD 280 nm
Δ Adrenal cell activi

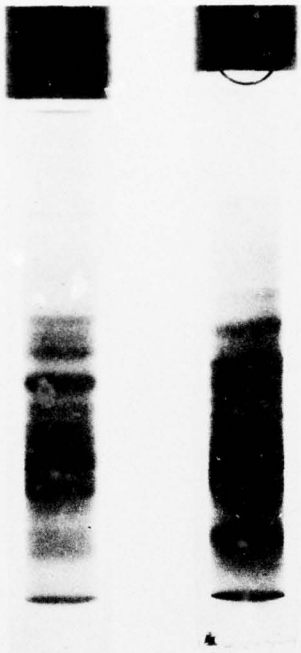


Figure 4. Standard acrylamide gel electrophoresis of soluble enterotoxin preparations.

Left: E. coli H197 soluble enterotoxin preparation (Sephadex G-75 void volume peak (Fig. 3) after chromatography of Sartorius SM11730 filtrate containing molecules <160,000 daltons)

Right: E. coli 74-114 soluble enterotoxin preparation (Sephadex G-75 void volume peak (Fig. 2) after chromatography of third Sephadex G-150 sol ECT peak)

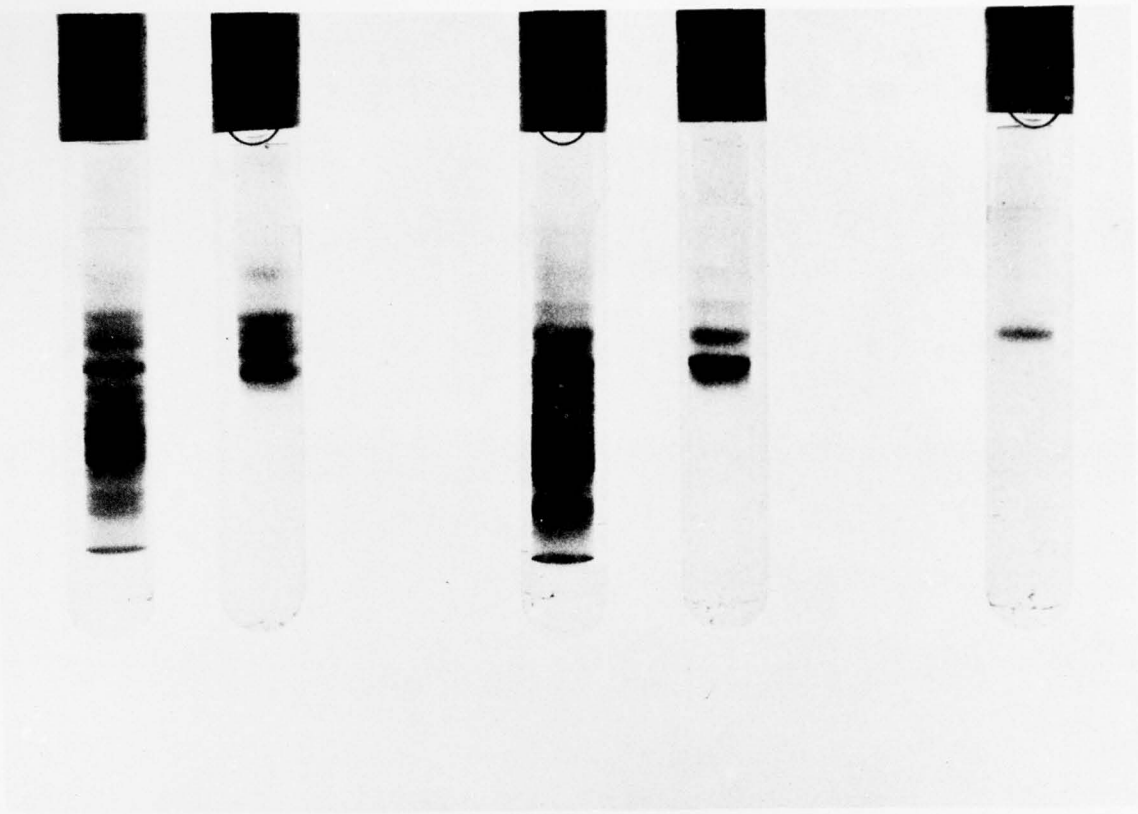


Figure 5. Standard acrylamide gel electrophoresis of soluble enterotoxin preparations before and after treatment with QAE-Sephadex A-25

From left to right:

1) E. coli H-197 soluble ECT preparation (Fig. 4) before QAE

2) E. coli H-197 soluble ECT preparation (Fig. 4) after QAE

3) E. coli 74-114 soluble ECT preparation (Fig. 4) before QAE

4) E. coli 74-114 soluble ECT preparation (Fig. 4) after QAE

5) PAGE-purified soluble ECT.

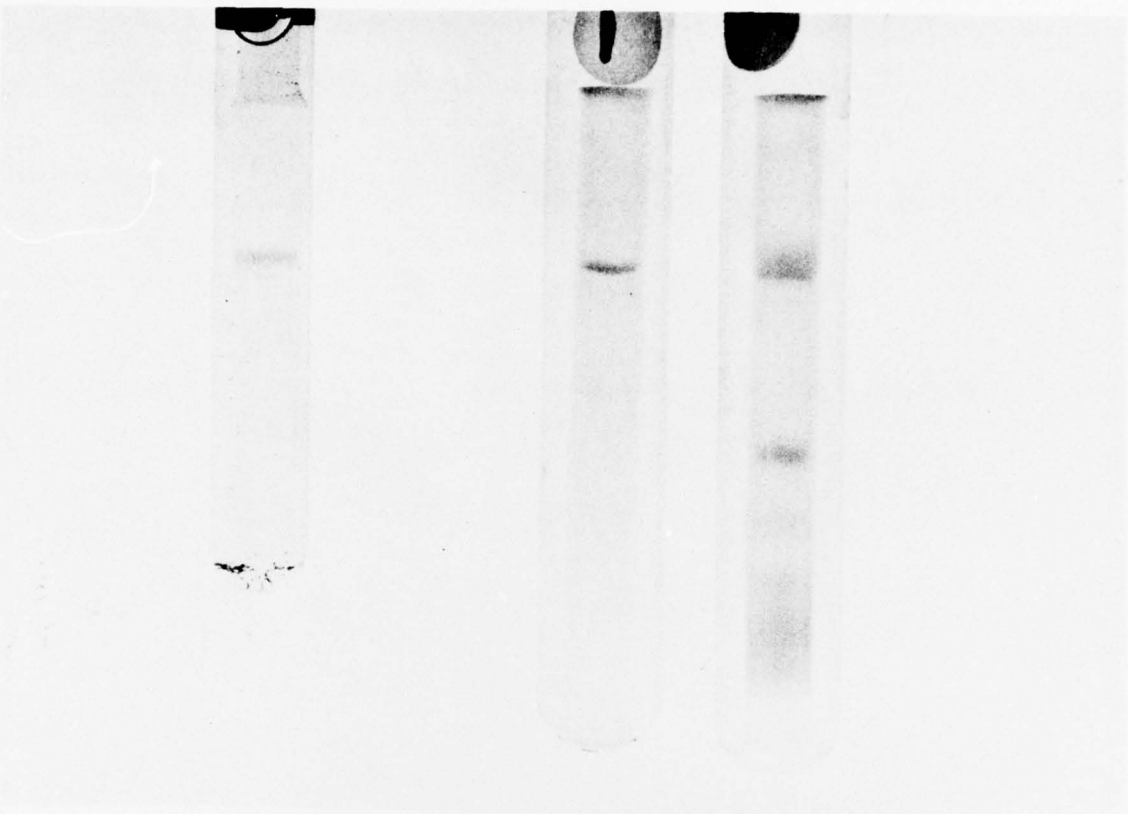


Figure 6. Standard and SDS acrylamide gel electrophoresis of PAGE-purified soluble enterotoxin from strain 74-114.

Left to right:

1) Standard gel of PAGE-purified sol ECT.

2) SDS gel of PAGE-purified ECT (in the presence of mercapto-ethanol and after boiling for 5 min.)

3) Same as (2) except with human serum albumin (≈70,000 daltons), carboxypeptidase (≈35,000 daltons) and lysozyme (≈14,000).

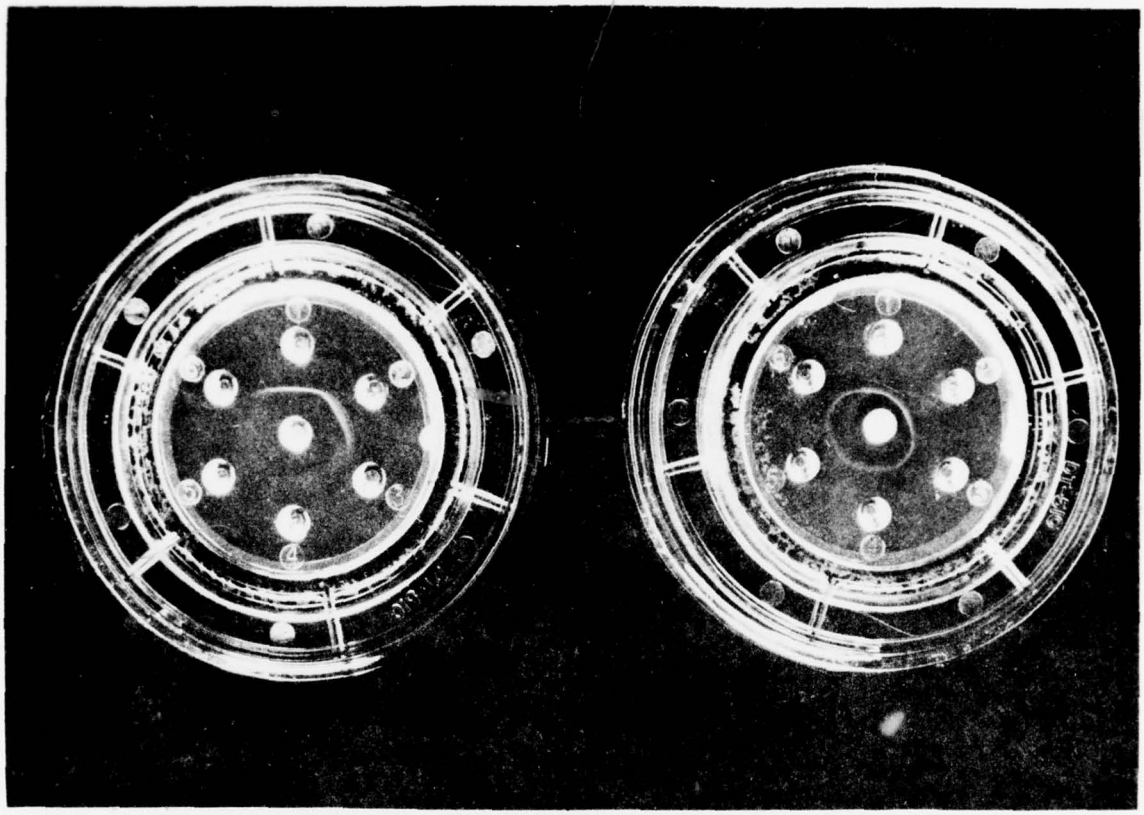


Figure 7. Double immunodiffusion of soluble E. coli enterotoxin and cholera toxin against E. coli antitoxin and cholera antitoxin.

Plates: Wells 1 & 2, cholera toxin (25 μg/ml)
Wells 3 & 4, E. coli 74-114, 3rd Sephadex G-150 peak after concentration and before QAE treatment -8 mg/ml
Wells 5 & 6, same as 3 & 4 except after one QAE treatment - 4.7 mg/ml.

Left plate: Cholera (goat #1) antitoxin, diluted 1:20

Right plate: E. coli (goat #2822) antitoxin undiluted

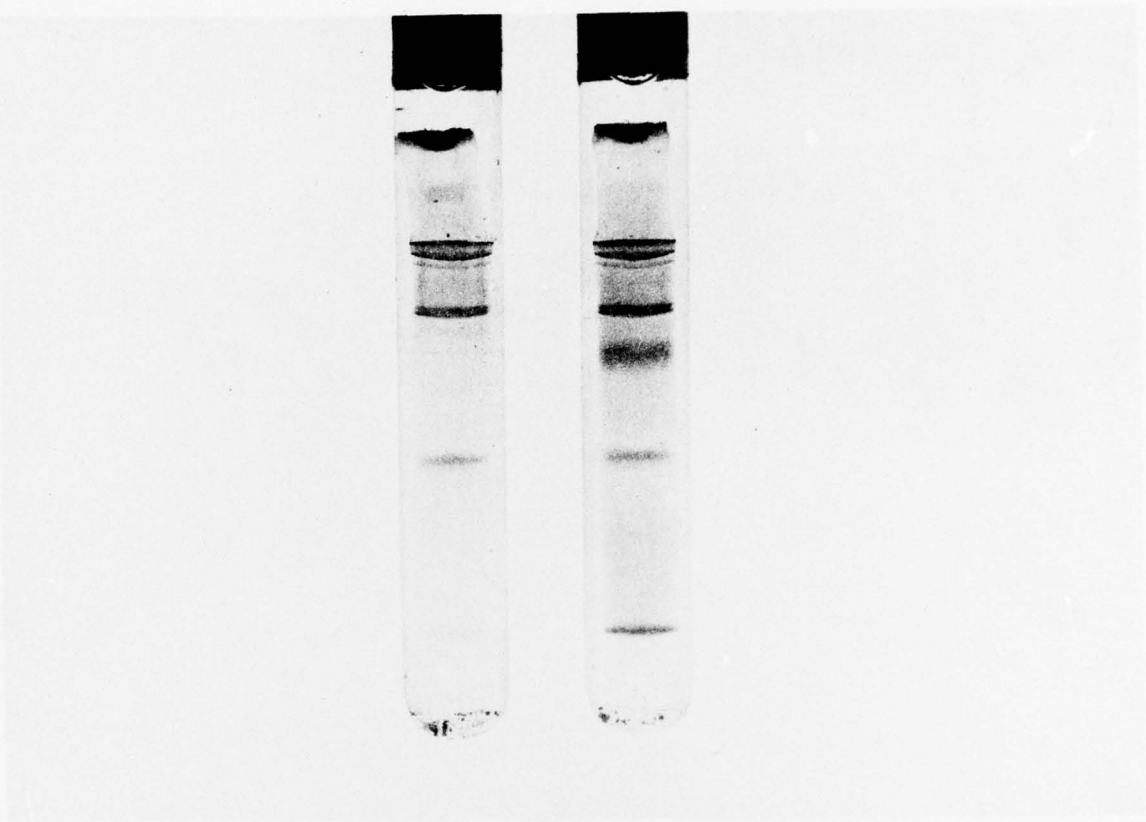


Figure 8. Standard acrylamide gel electrophoresis of insoluble enterotoxin from strain 74-114 before and after treatment with trypsin.

Sample: Insoluble ECT (I and II) from a Sephadex G150 column (Figure 1) after concentration by sedimentation (16 hr, 100,000 x g).

Left: Control - 100 µl/ml ammonium bicarbonate buffer, 37°C, 2 hr

Treated - 100 µl/ml trypsin (100 µg/ml, final concentration), 37°C, 2 hr

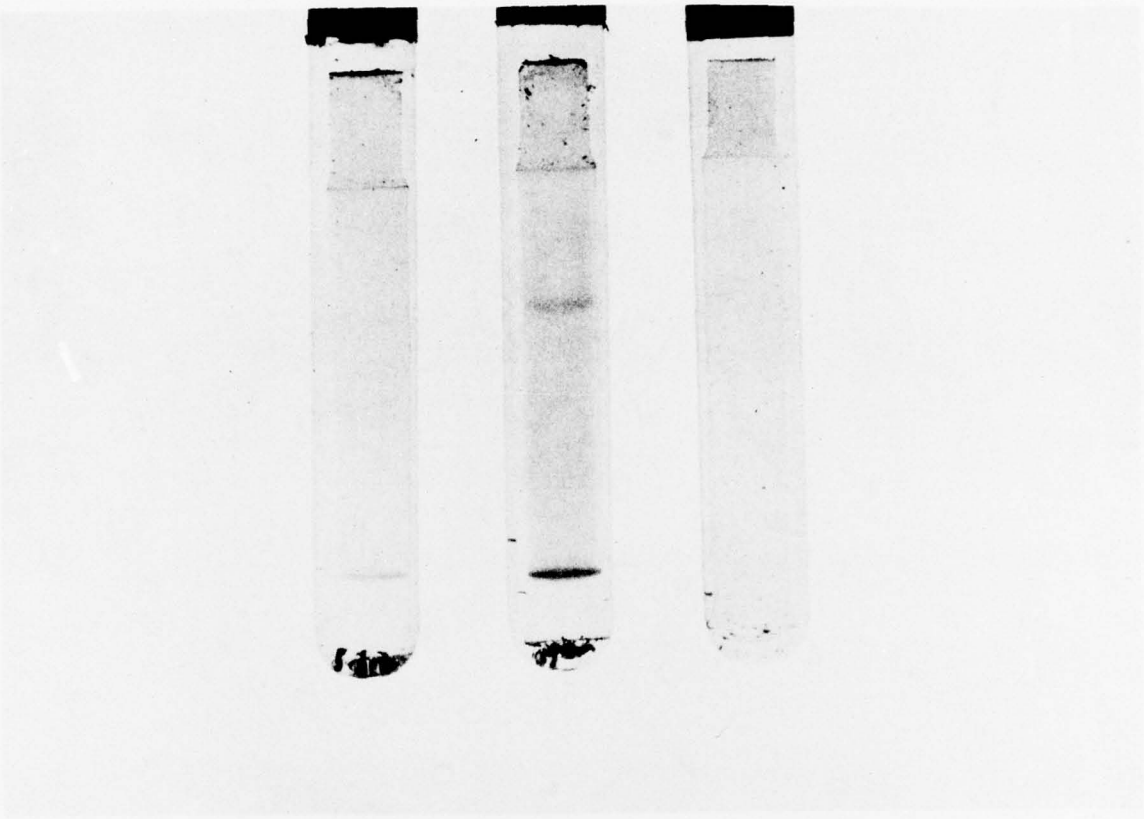


Figure 9. Standard acrylamide gel electrophoresis of "purified" insoluble enterotoxin (I) from strain 74-114 before and after treatment with trypsin.

Sample: low speed sediment of insoluble ECT preparation depicted in Figure 8.

1) Left: Control - 100 µl/ml ammonium bicarbonate buffer, 37°C, 2 hr

2) Treated - 100 µl/ml trypsin (100 µg/ml, final concentration), 37°C, 2 hr

3) Trypsin alone. 100 µg/ml, 37°C, 2 hr

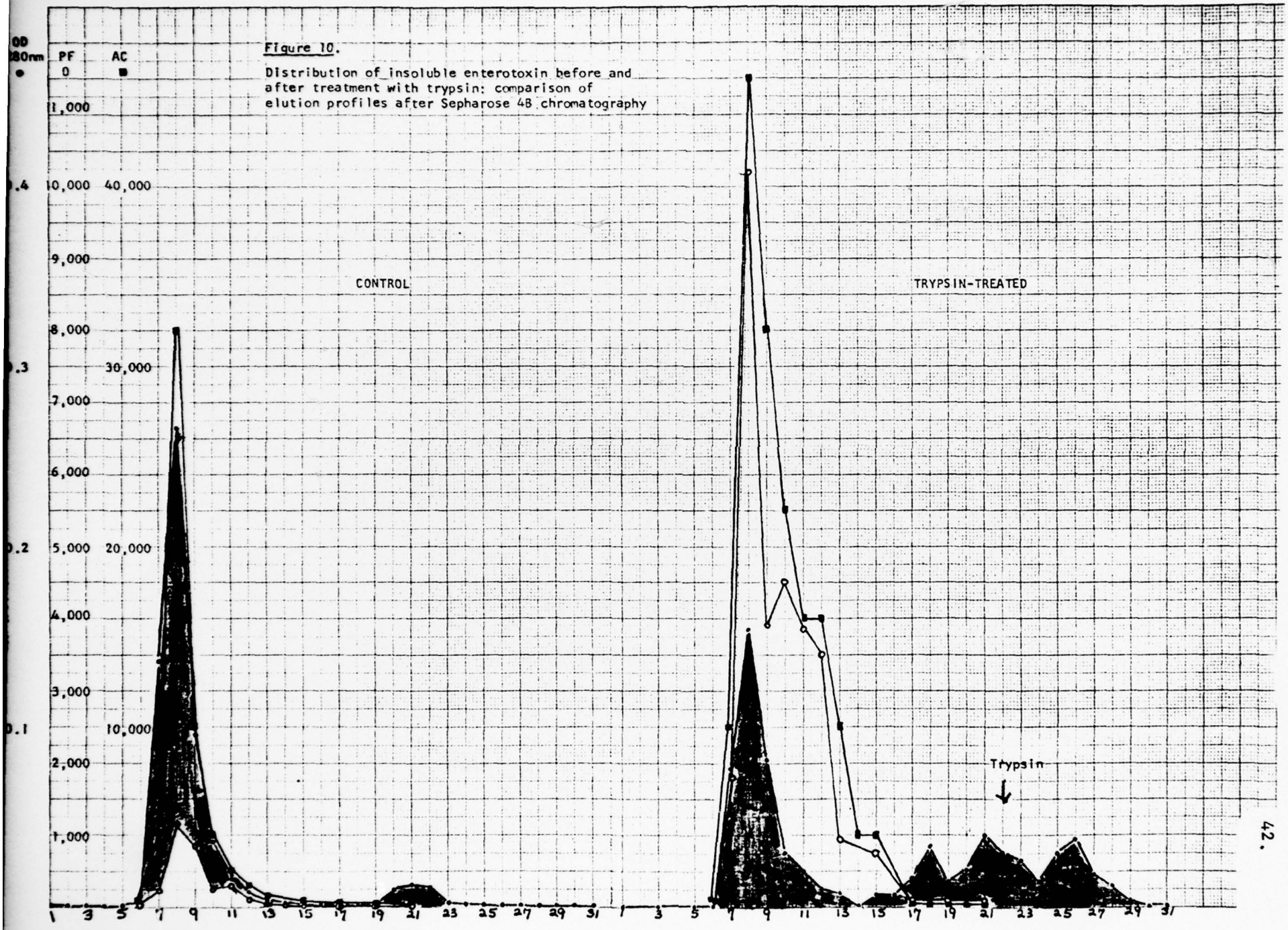


Figure 10.

Distribution of insoluble enterotoxin before and after treatment with trypsin: comparison of elution profiles after Sepharose 4B chromatography

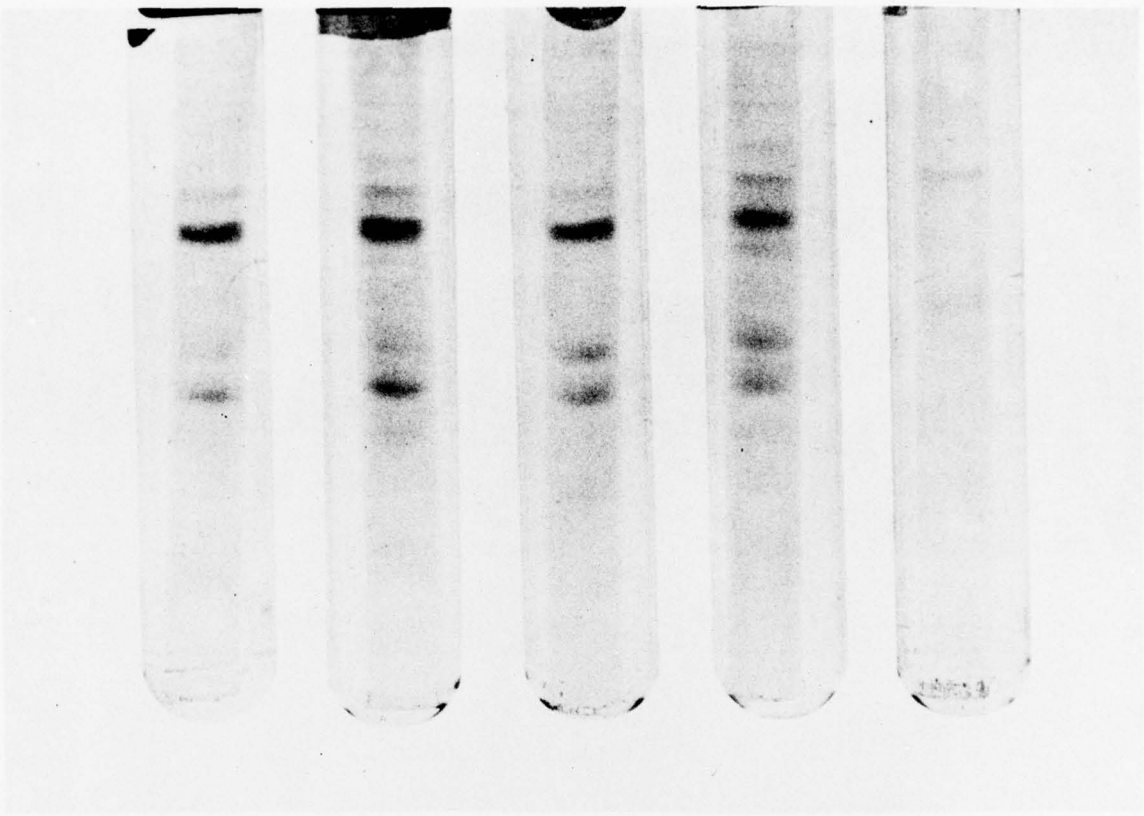


Figure 11. SDS Acrylamide gel electrophoresis of peak fractions after Sepharose 4B chromatography of control and trypsin-treated insoluble enterotoxin, respectively.

From left to right:

1) Control enterotoxin (strain 74-114) - Fr. #8, Sepharose 4B (Fig. 10), with mercaptoethanol and boiling 5 minutes

2) Control enterotoxin (strain 74-114) - Fr. #8, Sepharose 4B (Fig. 10) with no reducing agent, no boiling

3) Trypsin-treated enterotoxin (strain 74-114) - Fr. #8, Sepharose 4B (Fig. 10), with mercaptoethanol and boiling 5 minutes.

4) Trypsin-treated enterotoxin (strain 74-114) - Fr. #8, Sepharose 4B (Fig. 10), no reducing agent, no boiling.

5) PAGE-purified soluble enterotoxin (strain 74-114) with mercaptoethanol and boiling.

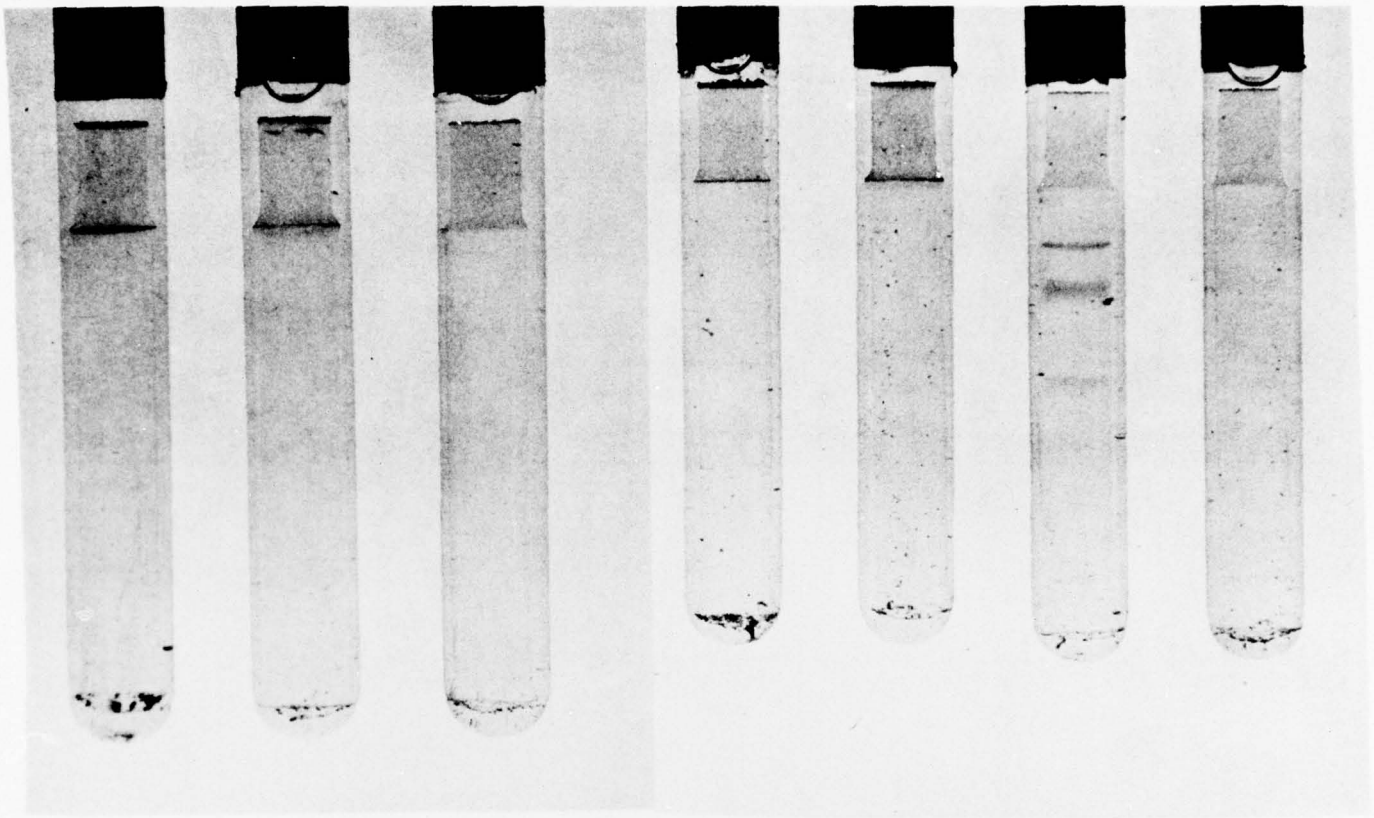


Figure 12. Standard acrylamide gel electrophoresis of fractions obtained after Sepharose 4B chromatography of control and trypsin-treated insoluble enterotoxin, respectively.

From left to right:

1) control enterotoxin (strain 74-114)-Sepharose 4B Fr. #8)
2) " " " " Fr.#11)See Fig.10
3) " " " " Fr.#21)

4) trypsin-treated enterotoxin (strain 74-114)-Sepharose 4B, Fr. #8)
5) ditto Fr. #10) See
6) " Fr. #18) Fig. 10
7) " Fr. #21)

Figure 13. Agarose A-50 M chromatography of twice sedimented insoluble enterotoxin from strain H197.

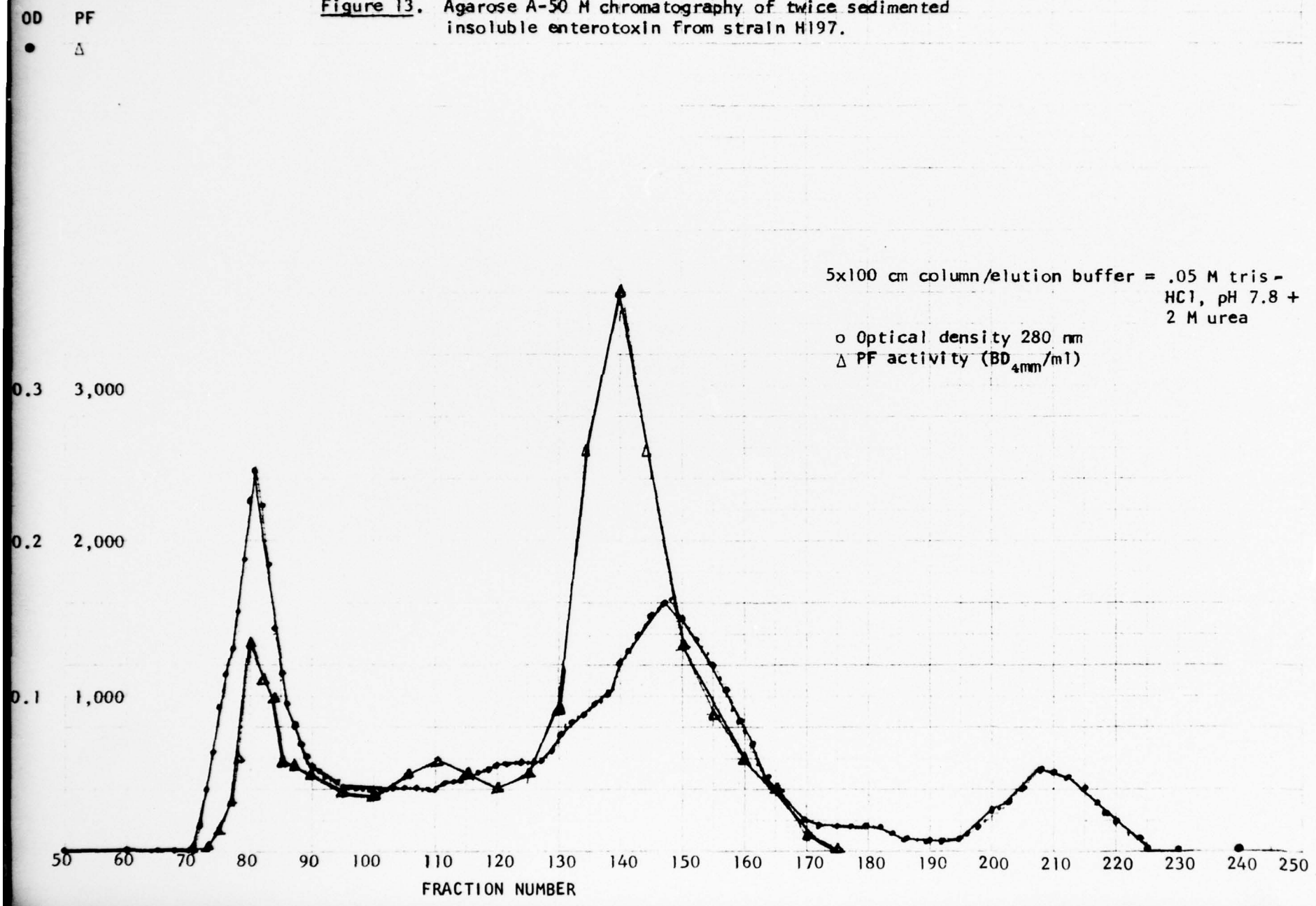


Figure 14. The effect of Lima Bean Trypsin Inhibitor on the secretory response to heat-labile enterotoxin from strain 74-114: comparison of various dilutions of culture filtrate administered with and without inhibitor.

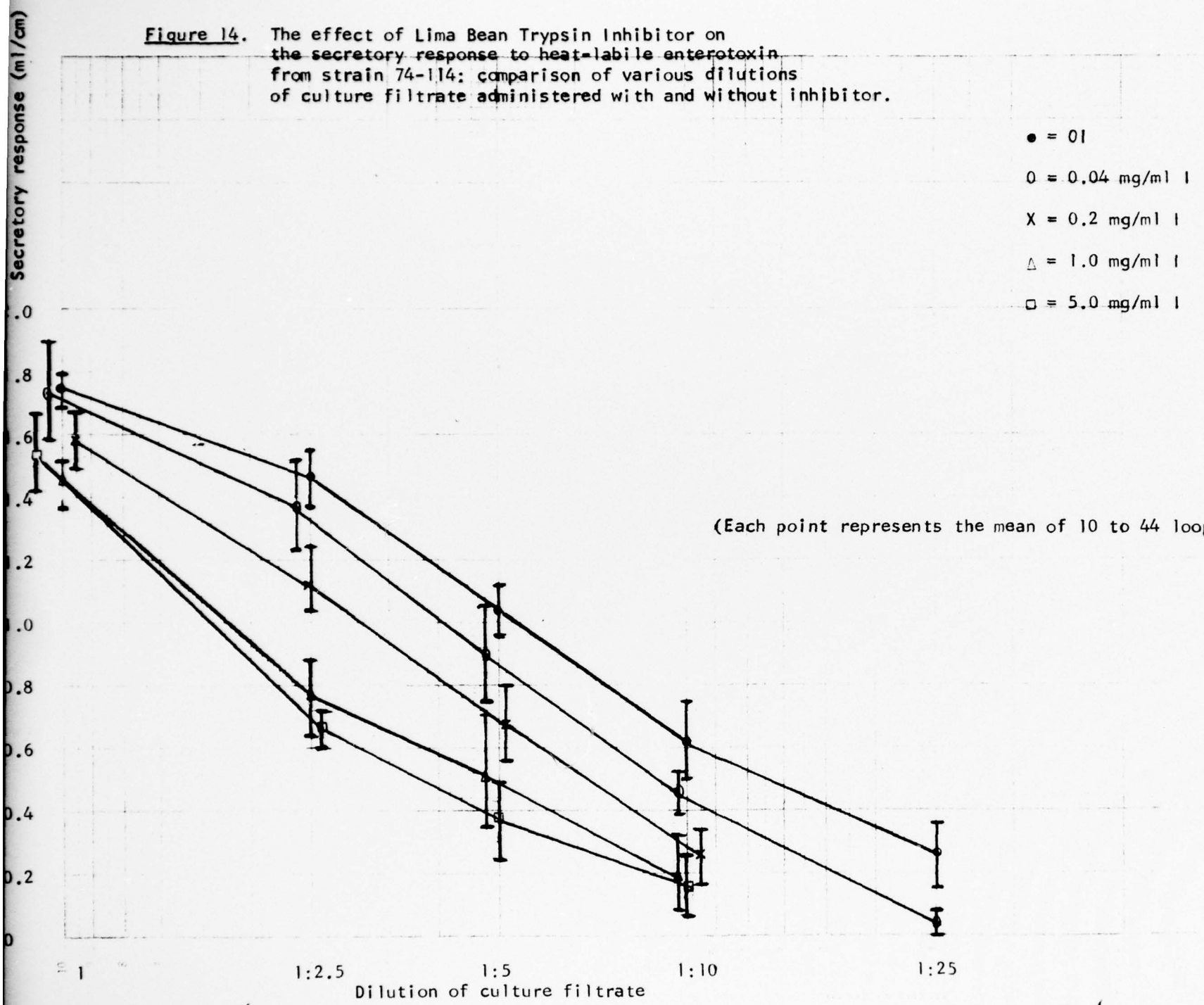
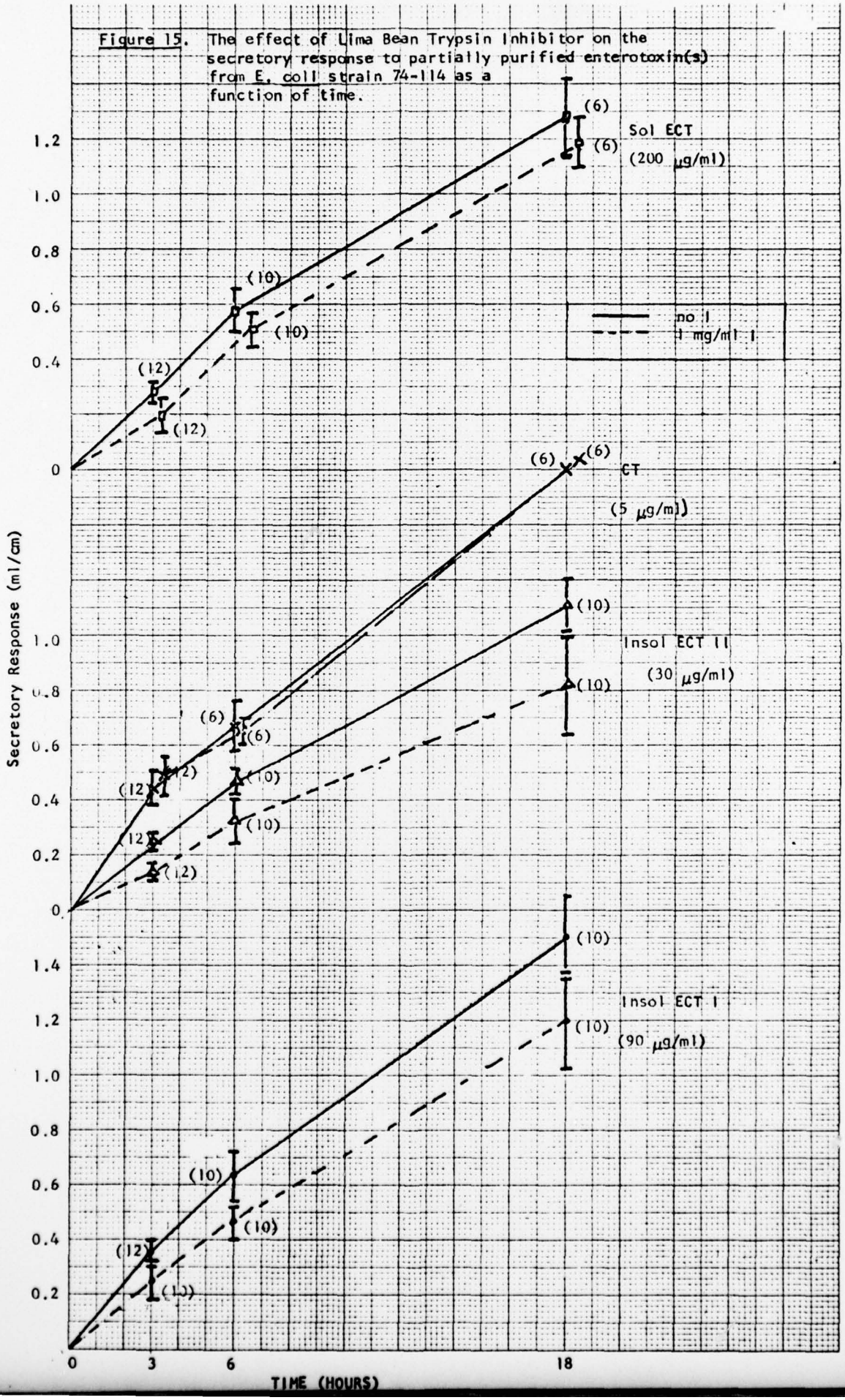


Figure 15. The effect of Lima Bean Trypsin Inhibitor on the secretory response to partially purified enterotoxin(s) from E. coli strain 74-114 as a function of time.



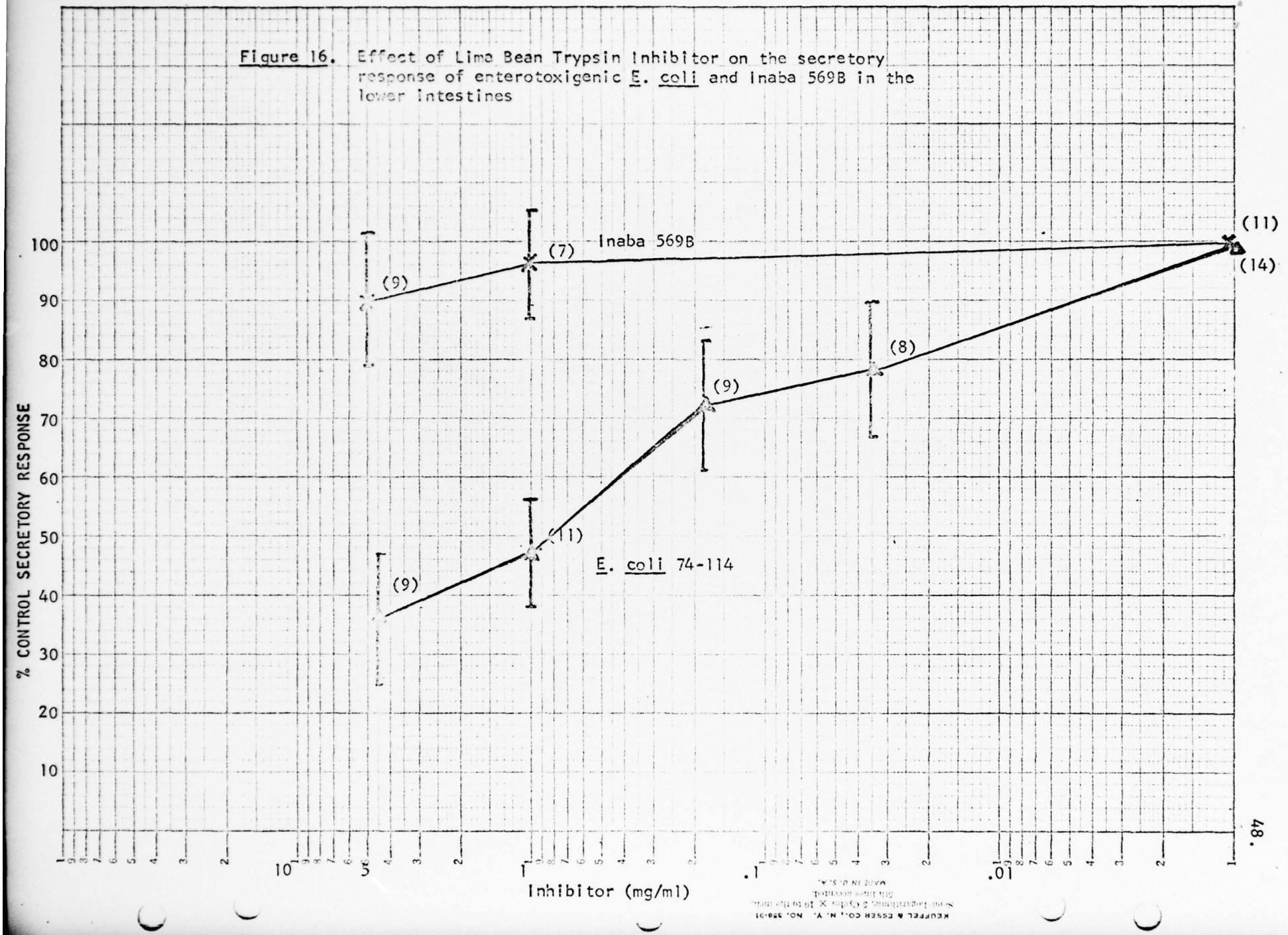


Figure 16. Effect of Lima Bean Trypsin Inhibitor on the secretory response of enterotoxigenic E. coli and Inaba 569B in the lower intestines

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