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<p>The goals of this research contract are to examine the primary action of <u>Shigella dysenteriae</u> 1 toxin (Shiga toxin) as an inhibitor of eukaryotic protein biosynthesis. Two major objectives of this investigation are designed to reveal Shiga toxin-induced changes in ribosomes structure-function relationships. These objectives are 1) to explain, in biochemical terms, the manner by which Shiga toxin enzymatically inactivates mammalian ribosomes and 2) to define the steps of protein biosynthesis which are specifically inhibited by the toxin as a result of ribosome modification. A comparison between Shiga toxin and similar fungus- and plant-derived toxins is to be made in regard to ribosome inactivation. It is likely that information obtained from these studies will be of value in describing the role of Shiga toxin in establishment of intestinal infections by the toxin-producing <u>Shigella</u> species. In this contract we have further delineated how</p>					
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MODE OF ACTION OF SHIGELLA TOXIN: EFFECTS ON  
RIBOSOME STRUCTURE AND FUNCTION

Final Report

Tom G. Obrig, Ph.D.

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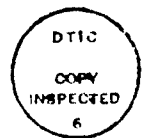
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Abstract (continued)

Shiga toxin inhibits peptide elongation in reticulocyte protein synthesis. More exactly, we have demonstrated that Shiga toxin inhibits peptidyl-tRNA translocation on ribosomes, a step catalyzed by protein synthesis elongation factor 2. However, from our previous data, we believe that Shiga toxin is a primary inhibitor of elongation factor 1 reactions (ie. aminoacyl-tRNA binding to ribosomes) and a secondary inhibitor of the elongation factor 2 reactions. In addition we have shown, by direct measurement, that Shiga toxin does not inhibit peptidyl transferase, an enzymatic center on 60S ribosomes which catalyzes peptide bond formation. Finally, during this contract our laboratory has identified a ribonuclease activity associated with Shiga toxin which hydrolyzes free ribosomal RNA in a defined and reproducible manner. Heat denaturation studies indicate similar sensitivity of the RNase and total protein synthesis inhibitory activities of Shiga toxin indicating that these properties are one in the same. This RNase activity is demonstrable when concentrations of toxin and rRNA approach a 1:1 molar stoichiometry. In addition, this RNase activity has the unusual property of being inhibited by  $\leq 2\text{mM}$   $\text{Mg}^{2+}$  as  $\text{MgCl}_2$ .

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## FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised, 1985).

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Abbreviations appearing include:

rRNA - ribosomal ribonucleic acid  
rprotein - ribosomal protein  
tRNA - transfer ribonucleic acid  
mRNA - messenger ribonucleic acid  
eEF 1 - eukaryotic elongation factor 1 which is the  
aminoacyl-transfer RNA binding factor  
eEF 2 - eukaryotic elongation factor 2 which is the translocase  
factor  
eIF - eukaryotic initiation factor  
Met-tRNA<sub>f</sub> - initiator methionyl-tRNA  
GTP - guanosine triphosphate  
DTT - dithiothreitol, a reducing agent  
polysome - a mRNA molecule with 3+ ribosomes attached  
80S ribosome - ribosome comprised of 60S and 40S subunits  
60S, 40S ribosome - large and small ribosomal subunits, respectively  
DOC-KCl ribosomes - ribosomes treated with 1% Deoxycholate and 0.5M  
KCl  
TCA - trichloroacetic acid

#### STATEMENT OF THE PROBLEM

1. Shiga toxin, as a documented inhibitor of mammalian protein biosynthesis, may specifically affect individual functional steps in the overall process.
2. The toxin, known to inactivate large ribosomal subunits, may interact with and modify a protein or RNA component of the ribosome.
3. Toxin-induced structural modification of the ribosome may be responsible for the change in ribosome protein biosynthetic activity.
4. Because the process of eukaryotic cytoplasmic protein biosynthesis is virtually identical in all mammalian cells and tissues, it is likely that information obtained from this study will be pertinent to Shiga toxin mode of action in intestinal tissues invaded by toxigenic Shigella.

#### TECHNICAL OBJECTIVES

1. Describe, in biochemical terms, how purified Shiga toxin inhibits mammalian protein biosynthesis.
2. Identify the individual functional steps of protein synthesis which are inhibited by Shiga toxin.
3. Determine if the toxin-induced alteration in sub-cellular ribosome function is due to a structural modification of the ribosome.
4. Compare data from above with existing information on plant-derived toxins which resemble Shiga toxin mode of action at ribosome level.

## BACKGROUND

It was established early on that a proteinaceous toxin is produced by the pathogen *Shigella dysenteriae* 1 (1). However, the relationship of the toxin and pathogenic properties of *Shigella* bacilli remains to be fully determined. During infection, *Shigella* penetrates the bowel epithelium and causes intestinal lesions (2). It has been suggested that a toxin produced by *Shigella dysenteriae* 1 is responsible for eliciting host responses to the pathogen (3,4). Host responses to *Shigella* include fluid production by ileal loops, in vitro, and diarrhea, in situ (4).

Moreover, a further understanding of Shiga action in the disease process would be aided by a series of investigations utilizing purified toxin. With partially purified toxin, several concepts pertinent to Shiga toxin function have become known. It appears that Shiga toxin is cytotoxic to several cell lines including HeLa human cervical carcinoma and WI-38 human fibroblasts (5-8). In addition, the relative activity of Shiga toxin in cell cultures is parallel to that in the rabbit ileum test system (6). From these data one might hypothesize that Shiga toxin is a non-selective agent which manifests its toxic properties on a wide array of cell and tissue types. However, recent reports indicate that toxin from *S. Shigae* exhibits high-affinity binding to a limited number of cell types (8,9). This strongly suggests that the toxin resembles other well-known microbial-derived toxins (i.e., diphtheria toxin, cholera toxin and *Pseudomonas* exotoxin A) which bind with high affinity ( $K_d = 10^{-10}$  M) to receptors on cells (10).

All of the above mentioned toxins appear to be potent inhibitors of protein biosynthesis in eukaryotic cells. It is generally accepted that clinical symptoms associated with these toxin-producing bacteria are, in part, a result of their effect on protein synthesis. To go one step further, it has been suggested that Shiga toxin also elicits different physiological responses in the host target by virtue of its ability to efficiently inhibit protein biosynthesis (11). Indeed, there is ample evidence indicating that Shiga toxin has, as its primary action, the inhibition of protein biosynthesis in whole animals (12) cell cultures (8,11) and cell-free lysates (11,14,22). It seems clear that ribosomes are the primary target of Shiga toxin and more recent results indicate that the large ribosomal subunit of eukaryotic cells are specifically affected (15). At this time, little is known about the ribosomal component which is modified by Shiga toxin. Data from our laboratory indicates that the toxin may be a ribonuclease (20,24).

It is conceivable that such information would be used to advantage in design of a mode of therapy for Shiga toxin based on information regarding the ribosomal substrate. In addition, these studies should lead to implementation of a new molecular assay for Shiga toxin which would have great sensitivity and specificity.

Shiga toxin has been purified to apparent homogeneity from *S. shigae* (9,16) and *S. dysenteriae* 1 (17,18). The holotoxin from both sources has an approximate molecular weight of  $M_r = 70,000$  (9,18). Structural analysis of *S. shigae* toxin (9) indicates its similarity to

cholera toxin; Shiga toxin has a single larger peptide of  $M_r = 30,000$  and multiple copies of a smaller peptide ( $M_r = 5,000$ ). It is also apparent that Shiga toxin resembles other multicomponent proteinaceous toxins (19) by having catalytic toxicity and cell binding specificity functions located on distinct peptides. That is, the larger peptide (subunit "A") is catalytic while smaller peptides (subunit "B"), appear to infer binding specificity properties of the holotoxin.

## METHODS

Activation of Shiga Toxin - Shiga toxin was purified from Shigella dysenteriae 1 strain 3818-0 as described by Brown, et al. (18) and activated, to increase in vitro potency by 70 to 100-fold (14), by adding 10 µg TPCK trypsin to a 1.0 ml solution containing Shiga toxin (0.42 mg/ml), 10 mM Tris-Cl, pH 8.0 and 100 mM NaCl. The mixture was incubated at 37°C/60 min. and 0.02 µg of phenylmethylsulfonyl fluoride was added to inactivate the trypsin. Urea and dithiothreitol were added to 8 M and 10 mM final concentrations, respectively, and incubated at 37°C/60 min. This sample was dialyzed vs. 3 x 100 vol of Type 1 water. Activated toxin was then lyophilized and stored at -70°C. Upon reconstitution in water, toxin prepared in this manner retained full activity as an inhibitor of cell-free protein synthesis.

Reticulocyte Lysates and Ribosomes - To obtain reticulocytes, New Zealand white rabbits (2-2.5 kg) were injected daily on days 1 through 4 with 0.25 ml/kg of 2.5% phenylhydrazine, pH 7.0 in 0.14 M NaCl. On the 7th day rabbits were sacrificed and blood drained from the heart into a freshly prepared ice-cold NKM (0.14 M NaCl, 0.03 M KCl, 0.002 M MgCl<sub>2</sub>) solution containing 200 units of heparin/ml. Reticulocytes as measured with methylene blue staining were found to represent >90% of the total cell population. This whole blood was filtered through cheesecloth, centrifuged at 1,000 x g/10 min and serum removed along with an upper "buffy coat" layer of cells. Packed reticulocytes were gently resuspended in 20 vol of NKM solution and centrifuged as above. This washing procedure was repeated a total of three times.

Lysate was prepared with the addition of 1 vol of Type I water to packed cells followed by gently shaking (4°C/10 min) and centrifugation at 20,000 x g /15 min. Aliquots of the resultant supernatant were stored at -80°C for up to 1 year without loss of activity. When first employed, each batch of lysate was tested with varying concentrations of hemin and magnesium acetate to determine concentrations required for maximum protein synthetic activity. Rates of protein synthesis in the lysate system were very close to that of whole reticulocytes.

Ribosomes were prepared from reticulocytes by washing with deoxycholate, then with 0.5 M KCl and will be referred to as DOC-KCl ribosomes. Ribosomal subunits were obtained by dissociation of polysomal 80S monomers in the presence of 0.5 M KCl and 1 mM puromycin (23). Subunits were separated with 35 ml 10-30% sucrose gradients by centrifugation in a SW27 rotor (Beckman) at 13,500 rpm/16 h/4°C and precipitated by addition of magnesium chloride to 10 mM and 0.7 vol. 95% pre-cooled (-20°C) ethanol. Precipitated ribosomes were collected by centrifugation at 6,000 x g/20 min/4°C, resuspended and stored at -70°C.

Purification of eEF-1 and eEF-2 from Rabbit Reticulocytes - eEF-1 and eEF-2 was isolated from the 100,000 x g supernatant of reticulocyte lysate. The purification procedure included sequential steps of ammonium sulfate fractionation, gel filtration, DEAE-cellulose, hydroxylapatite and Cm-cellulose chromatography. In some cases, purified elongation factors from wheat germ supplied by Dr. J. Ravel (U. Texas at Austin) were utilized and found to be comparable in activity to the reticulocyte proteins.

[<sup>3</sup>H] Phe-tRNA Synthesis - Aminoacylation of yeast tRNA with [<sup>3</sup>H]Phe was carried out in 4.0 ml reaction containing: 100 mM Tris-Cl, pH 7.4, 10 mM Mg(OAc)<sub>2</sub>, 10 mM dithiothreitol, 2 mM ATP, 2.6 mg creatine phosphate, 0.1 mg creatine phosphokinase, 200 μg of reticulocyte ribosomal 0.5 M KCl wash protein, 5 mg tRNA, and 100 μg [<sup>3</sup>H]Phe (SA=2,000 Ci/mol). The reaction was incubated at 37°C/40 min and monitored for cold 10% trichloroacetic acid-insoluble radioactivity precipitated onto glass fiber filters. [<sup>3</sup>H]Phe-tRNA was extracted by addition of 2.0 M K(OAc), pH 5.0 to a final concentration of 0.1 M and 1 vol phenol saturated with 10 mM K(OAc), pH 5.0. Following agitation for 15 min/4°C and centrifugation, the aqueous layer was removed and [<sup>3</sup>H]Phe-tRNA precipitated by addition of 2.5 vol of 95% ethanol at -20°C/2 h. The [<sup>3</sup>H]Phe-tRNA pellet was washed with 95% ethanol and then with ethyl ether to remove residual phenol.

eEF-1 and eEF-2 GTPase Assay - The eEF-2 GTPase reaction (21) mixture was performed in a total volume of 50 μl containing, in order of addition: 25 mM Tris-Cl, pH 7.4, 100 mM KCl, 5 mM Mg(OAc)<sub>2</sub>, 2.5 mM dithiothreitol, Shiga toxin as indicated, 1.0 A<sub>260</sub> units DOC-KCl washed ribosomes, 0.2 μg eEF-2, and 5 μM [γ-<sup>32</sup>P]GTP (SA=1,000 Ci/mol). The reaction mixture was incubated at 37°C/10 min, stopped by addition of 0.25 ml 0.02 M silicotungstic acid in 0.02 N H<sub>2</sub>SO<sub>4</sub>, 0.5 ml 1 mM potassium phosphate, pH 5.8, and 0.25 ml of 5% (W/V) ammonium molybdate in 4 N H<sub>2</sub>SO<sub>4</sub>. The phosphomolybdate was extracted into 1.0 ml of isobutanol/benzene (1:1 v/v), centrifuged at 500 x g for 5 min and radioactivity of 0.5 ml of the aqueous phase was monitored in a scintillation counter.

eEF-2 Translocation Assay - [<sup>3</sup>H]Phe-tRNA was non-enzymatically bound to DOC-KCl washed ribosomes in a batch reaction containing the following, in a final total vol of 52 ml: 50 mM Tris-Cl, pH 7.4, 120 mM KCl, 16 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 10 mg poly(U), 620 A<sub>260</sub> units of DOC-KCl washed ribosomes, and 3 nmol of [<sup>3</sup>H]Phe-tRNA (SA=1500 Ci/mol). The reaction mixture was incubated at 37°C/20 min and chilled on ice for 10 min. Approx. 26 ml of the reaction mixture was layered over 7 ml of a 15% sucrose solution containing 50 mM Tris-Cl, pH 7.4, 120 mM KCl, 8 mM MgCl<sub>2</sub> and 5 mM 2-mercaptoethanol in a 35 ml tube. The contents were centrifuged in a SW-27 rotor (Beckman) at 24,000 rpm/16h/4°C. Supernatants were decanted and the pellets resuspended in 20 mM Tris-Cl, pH 7.4, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM 2-mercaptoethanol and 10% glycerol at 125 A<sub>260</sub> units/ml.

The translocation assay contained the following listed in order of addition, in a final 0.5 ml vol: 50 mM Tris-Cl, pH 7.4, 70 mM KCl, 5 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 3.0 A<sub>260</sub> units DOC-KCl washed ribosomes with [<sup>3</sup>H]Phe-tRNA (5,000 cpm) non-enzymatically bound as described above, activated Shiga toxin as indicated, 0.5 μg eEF-2 protein, and 0.2 mM GTP. The reaction mixture was incubated at 37°C/6 min and cooled to 4°C. Puromycin-HCl was added to 1 mM and incubated at 4°C/20 min. [<sup>3</sup>H]Phe-puromycin was extracted from the reaction mixture by addition of 0.5 ml 2 M ammonium bicarbonate, pH 9.0 and 1.0 ml ethyl acetate. A portion of the organic phase was monitored for radioactivity in 10 ml of scintillation fluid.

[eEF-2·GTP·80S ribosome] Complex Formation The 100  $\mu$ l reaction mixture contained the following components listed in order of addition: 25 mM Tris-HCl, pH 7.4, 110 mM K(OAc), 5 mM Mg(OAc)<sub>2</sub>, 2.5 mM dithiothreitol, 1.0 mM DCC, 10 unit DOC-KCl washed ribosomes (21 pmol), 0.4  $\mu$ g eEF-2 protein, Shiga toxin as indicated, and 1  $\mu$ M [<sup>3</sup>H]GTP (SA=5,000). The reaction was incubated at 37°C/10 min, terminated by addition of 6 ml ice cold Solution A, and collected on a BA35, 0.45  $\mu$  pore size nitrocellulose filter. Filters were washed with 3 x 15 ml ice cold Solution A, placed in vials containing 5 ml aqueous scintillant, shaken for 60 min/4°C and counted.

Ribonuclease Assay Total rRNA, extracted from 60S ribosomes with phenol-chloroform, was separated into 28S, 5.8S and 5S species on preparative 5% polyacrylamide gels in the presence of 8M urea. The 5.8S or 5S rRNA were extracted from gels and rerun to obtain purified RNA samples. Throughout, caution was taken to utilize RNase-free labware, reagents and type I water.

Toxins were incubated with either 5S or 5.8S rRNA in water at 37°C/10min. Concentrations of toxins are noted in each figure. Following incubation, rRNA was 3' end-labeled at 4°C/12hr. with [<sup>32</sup>P-5']pCp, S.A.=2500 Ci/mmol, and T4 ligase as described by Peattie (13). The sample was precipitated with 70% ethanol and dissolved in electrophoresis sample buffer consisting of: 8M urea, 20mM Tris-HCl, pH 7.4, 1mM Na<sub>2</sub>-EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue. Samples<sup>2</sup> (5 $\mu$ l) were heated (65°C) and layered onto a 0.75mm thick 10% polyacrylamide gel containing 7M urea and electrophoresed at 300v/4.5hr/10°C in a Tris-borate, pH 8.3 buffer system containing EDTA. Autoradiography was performed using X-OMAT (Kodak) film.

## RESULTS AND DISCUSSION

### Shiga toxin does not inhibit initiation of mammalian protein biosynthesis

Because Shiga toxin was known to selectively inactivate 60S ribosomes (15), and the 60S ribosome is required in the final step of eukaryotic protein synthesis initiation (Fig. 1, step IV), we performed a detailed study to determine if Shiga toxin affected initiation of eukaryotic protein biosynthesis. Results from this series of experiments were conclusive; Shiga toxin did not interfere with formation of intermediate complexes or reactions presented in the following scheme.

These studies were conducted in either reticulocyte lysates with endogenous mRNA or in assay mixtures containing partially purified initiation factors, ribosomes, artificial mRNA and initiator [<sup>35</sup>S]methionyl-tRNA. These components were prepared in Dr. Obrig's laboratory using established procedures. Results from one such experiment is presented (Table 1). The basis of this assay is formation of the first complex of initiation resulting from step 4 (Fig. 1) and its reaction with puromycin to yield [<sup>35</sup>S]Met-puromycin as indicated in step 5. Shiga toxin exhibited only a marginal effect on this assay, indicating the toxin did not inhibit steps number 1 through 5, i.e., the entire peptide initiation process.

As expected, other steps shown in Fig. 1, when measured individually were not inhibited by a concentration of Shiga toxin which was shown to inhibit overall protein synthesis. For example, step 1 in Fig. 1 is formation of a ternary complex among [<sup>35</sup>S]Met-tRNA, initiation factor eIF-2 and GTP. The complex is collected on a nitrocellulose filter while individual components pass through the filter. Shiga toxin had no effect on this reaction (Fig. 2).

During initiation of protein synthesis, the ternary complex discussed above binds to 40S ribosomal subunit and is further stabilized by addition of mRNA to the growing complex (see Fig. 1). Formation of the pentameric complex was accomplished by supplementing the previous reaction mixture (for 3' complex) with purified 40S ribosomal subunits and the artificial mRNA, poly AGU which contains the AUG initiation codon. Incorporation of [<sup>35</sup>S]Met-tRNA<sub>f</sub> into the pentameric initiation complex was monitored by its retention on nitrocellulose filters as described for the previous assay. The 3' initiation complex was very short-lived in the presence of Mg<sup>2+</sup> (added to this reaction mixture as magnesium acetate) which prevented interference of 3' complex formation with measurement of the pentameric initiation complex with the filter assay procedure. Our results indicated that Shiga toxin, at 1 µg/ml, did not appreciably alter formation of the pentameric initiation complex (Table 2).

The effect of Shiga toxin on pentameric initiation complex formation was also monitored in unfractionated reticulocyte lysates. Rationale for this assay procedure follows. Pentameric initiation complex was accumulated in reticulocyte lysate in response to added NaF and β, γ-methylene GTP which inhibited the subsequent initiator step #4 (Fig. 1), i.e., addition of 60S ribosomal subunits to the pentameric complex. If Shiga toxin activity inhibited one or more of the steps preceding step #4 in Fig. 1, one should see a reduced amount of pentameric complex formed.

In this case, the sucrose gradients were employed for quantitative and qualitative analysis of [<sup>35</sup>S]Met-tRNA<sub>f</sub> binding into the pentameric initiation complex. Results from this assay (Table 3) were in full agreement with those obtained with the filter assay procedure. It was concluded that Shiga toxin did not inhibit steps #1 through #3 in Fig. 1.

#### Shiga toxin is a potent inhibitor of the peptide elongation process

Evidence suggesting a lesion at peptide elongation was suggested by polysome accumulation data (14,15). Using highly purified reticulocyte factors eEF1, eEF2, ribosomes etc., we have determined which of the three steps of peptide elongation are inhibited by Shiga toxin. These steps are 1) eEF1-dependent binding of aminoacyl-tRNA into the ribosomal A-site 2) peptide bond formation between aminoacyl-tRNA molecules in the ribosomal and P sites catalyzed by peptidyl transferase and 3) eEF2-dependent translocation of aminoacyl-tRNA from the ribosomal A-site to the P-site. Our results were conclusive, Shiga toxin is a primary inhibitor of eEF1-dependent reactions, a secondary inhibitor of eEF2-dependent translocation and has no effect on peptidyl transferase activity. Data that support these conclusions are the following.

#### Shiga toxin specifically inhibits aminoacyl-tRNA binding to ribosomes

This series of experiments measured elongation factor-1 (EF1)-dependent binding of [<sup>3</sup>H]phe-tRNA to isolated reticulocyte ribosomes in the presence or absence of Shiga toxin. The phe-tRNA binding was GTP and poly(U)-dependent and was catalyzed with purified eukaryotic EF1 protein. The data indicate Shiga toxin has a clear effect on this reaction (Fig. 3). Interestingly, the potency of Shiga toxin against phe-tRNA binding is virtually identical to its relative inhibitory activity for overall protein synthesis as measured by [<sup>3</sup>H]leucine incorporation into globin protein in unfractionated reticulocyte lysates (Fig. 3).

Other data support the concept that the primary action of Shiga toxin is on EF1-catalyzed reactions which require intact ribosomes. The GTPase activity associated with EF1 protein was also inhibited by Shiga toxin in a dose-dependent manner (Fig. 4). These effects of Shiga toxin result from its action on 60S ribosomal subunits rather than on EF1 directly as excess EF1 protein could not overcome the inhibition.

#### Shiga toxin, secondarily, inhibits eEF2-dependent translocation on ribosomes

Shiga toxin inhibits the coupled eEF2 translocation and peptidyl transferase reaction. This "coupled" reaction is presented schematically in Fig. 5. When this reaction was carried out in the presence of Shiga toxin, formation of [<sup>3</sup>H]Phe-puromycin was inhibited in a dose-dependent manner (Fig. 6). However, a 30-fold higher concentration of Shiga toxin was required to inhibit this reaction by 50% than was required to inhibit overall protein synthesis by 50% in a reticulocyte lysate (Fig. 6) or eEF Phe-tRNA binding by 50% (Fig. 3).

We also examined the effect of excess eEF-2 protein on the translocation reaction. Excess eEF-2 protein completely reversed Shiga

toxin inhibition of the translocation reaction (Fig. 7). Translocation reactions were saturated by 0.6  $\mu\text{g}$  eEF-2 protein in the absence of toxin, but in the presence of Shiga toxin required 6.0  $\mu\text{g}$  of eEF-2 protein to reach plateau level. These data suggest that Shiga toxin may alter the affinity of ribosomes for eEF-2 protein, a phenomenon which is completely overcome by excess eEF-2 protein. In this translocation reaction, it was also determined that varying the KCl concentration over a 10 to 130mM range had only a slight effect on Shiga toxin's ability to inhibit [ $^3\text{H}$ ]Phe-puromycin formation. Thus, in the presence of limiting eEF-2 protein, increasing the rate of translocation by increasing the KCl concentration, had little effect on Shiga toxin ability to prevent eEF-2 interaction with ribosomes (Fig. 8.).

#### Shiga toxin does not inhibit eEF-2·GTP·80S ribosome complex formation.

Another means of measuring eEF-2 interaction with ribosomes is to monitor formation of the 3' ribosomal complex comprised of GTP, eEF-2 protein and ribosomes. Both aminoacyl-tRNA and mRNA are omitted from this assay. Shiga toxin at concentrations up to 1  $\mu\text{M}$  did not affect complex formation (Table 4). Thus, Shiga toxin appears to differ from other ribosome-inactivating toxins such as phytolectin and abrin which are strong inhibitors of 3' complex formation.

#### Shiga toxin does not inhibit eEF-2 GTPase.

The effect of Shiga toxin on eEF-2 dependent GTPase activity was examined over a concentration range of 0.1 nM to 1  $\mu\text{M}$  toxin. This GTPase activity is considered to be "uncoupled" as it was carried out in the presence of ribosomes, GTP and eEF-2 protein but in the absence of aminoacyl-tRNA and mRNA. Although 50% inhibition of lysate protein synthesis occurs at 6.4 nM Shiga toxin, only slight inhibition of GTPase activity was observed at concentrations of up to 1  $\mu\text{M}$  toxin. As observed with inhibition of the translocation reaction, excess eEF-2 protein also reversed the minimal effect of the toxin on eEF-2 GTPase activity (Fig. 6).

#### Shiga toxin is without effect on the isolated peptidyl transferase reaction.

To test whether Shiga toxin inhibited peptidyl transferase we employed an assay in which the eEF-2-dependent translocation and peptidyl transferase reactions proceed in two separate, but sequential steps (Fig. 5). [ $^3\text{H}$ ]Phe-tRNA is bound non-enzymatically into the ribosomal A-site. In the two step uncoupled reaction, the first step involves the eEF-2-dependent movement of [ $^3\text{H}$ ]Phe-tRNA on ribosomes for the A- to P-site. This carried out at 37°C in the absence of puromycin. Then the reaction mixture is reduced to 4°C. The second incubation involves reaction by peptidyl transferase<sub>3</sub> of the [ $^3\text{H}$ ]Phe-tRNA located in the P-site with puromycin to yield [ $^3\text{H}$ ]Phe-puromycin. Only the peptidyl transferase reaction proceeds at 4°C, i.e. the translocation reaction requires a higher temperature than 4°C. Thus, if one adds the toxin during the second step, one can observe its direct effect on the peptidyl transferase. When 1  $\mu\text{M}$  Shiga toxin was present only during the peptidyl transferase step (i.e. 2nd incubation), no effect on that activity was detected (Table 5).

## Shiga toxin inhibits the isolated translocation reaction.

Shiga toxin (1 $\mu$ M) added during the translocation step (Fig. 5) reduced [<sup>3</sup>H]Phe-puromycin formation to 45% of control values (Table 5). Puromycin was absent from the reaction, thus only translocation was being measured. Subsequent to this incubation, puromycin was added and the second i.e. peptidyl transferase was carried out at 4°C. Translocation does not occur at 4°C. Other inhibitors of the translocation reaction, including alpha-sarcin, phytolectin and cycloheximide, also inhibited translocation while exhibiting only a marginal direct effect on peptidyl transferase activity (Table 5).

## How Does Shiga Toxin Inactivate 60S Ribosomes?

This is a major question which remains unanswered at the completion of this contract.

## Shiga toxin does not modify ribosomal proteins

Numerous attempts were made to reveal a Shiga toxin-related change in one or more of the 60S ribosomal subunit proteins. These proteins were isolated from either toxin-treated or control ribosomes and analyzed by 2-D PAGE. Separation in the first dimension was by net charge, and in the second dimension by protein mass. Results of typical r-protein patterns are presented in Figure 9. Although we conclude that no toxin-related changes occur in these parameters, it is possible that Shiga toxin could modify the r-proteins in a way which would not be detectable by this 2-D PAGE procedure.

## Shiga toxin does not exhibit RNase activity similar to alpha-sarcin

Data accumulated to date indicate that Shiga toxin does not hydrolyze rRNA of intact ribosomes to yield a rRNA fragment as does alpha-sarcin, a known 60S ribosomal subunit inactivator and specific RNase. A series of experiments were conducted to determine if Shiga toxin possesses RNase activity which could explain how it inactivates 60S ribosomal subunits. Alpha-sarcin was included as a positive control as this fungal-derived toxin is known to specifically hydrolyze rRNA of intact ribosomes to yield an approximate 500 nucleotide fragment from the 3'-terminus of 28S rRNA. Results from one such experiment are presented (Fig. 10). The alpha-sarcin cleavage fragment is designated  $\alpha$ -s. Shiga toxin action did not result in the formation of  $\alpha$ -s or any other rRNA fragment not present in the control samples. These results were unchanged if ribosomal RNA from control and toxin-treated ribosomes were analyzed on PAGE by silver staining or by autoradiography following 3'-end labeling with [5'-<sup>32</sup>P]pCp and T<sub>4</sub> RNA ligase.

## Shiga Toxin Exhibits Ribonuclease Activity on Free rRNA Substrate

The results are described below of a study which was conducted to determine if Shiga toxin possesses RNase activity. The purified protein was incubated with isolated 5.8S rRNA of 60S ribosomes. The RNA products were then 3'-end labeled with [<sup>32</sup>P-5']pCp and T<sub>4</sub> ligase, subjected to electrophoresis in urea-polyacrylamide gels and analyzed following autoradiography. The results indicate that Shiga toxin (ST) exhibits a concentration-dependent RNase activity (Fig. 11). It was also determined

that this activity was time-dependent as incubation of  $10^{-6}$  M Shiga toxin with the 5.8S rRNA resulted in extensive degradation of the RNA within 30min. (Fig. 11, lanes 3-5). The RNA fragment patterns different for Shiga toxin and alpha-sarcin incubation reactions (Fig. 11, lane 1 vs. 3 or 4). A study was also performed to determine if the RNase and protein synthesis inhibitory activities of Shiga toxin exhibited similar heat denaturation properties. Preincubation of Shiga toxin at 70°C decreased its ability to hydrolyze 5.8S rRNA (Fig. 12). In a separate reticulocyte lysate protein synthesis study it was also revealed that the toxin lost its inhibitory activity at 70-75°C, but retained full activity following preincubation at 55°C. We conclude from these data that Shiga toxin may be a RNase. The conditions for this RNase activity on naked rRNA substrates have two requirements. First, a near equimolar concentration of both toxin and RNA are necessary. Second, magnesium (as  $MgCl_2$ ) is strongly inhibitory for the toxin-associated RNase activity.

## CONCLUSIONS

In summary, our cumulative results show that:

- 1) Shiga toxin is a potent inhibitor of eukaryotic protein synthesis at the level of peptide elongation.
- 2) The toxin is without effect on peptide initiation.
- 3) Shiga toxin is a primary inhibitor of elongation factor 1-dependent reactions on ribosomes. These reactions include aminacyl-tRNA binding to the ribosomal A-site and eEF-1 GTPase.
- 4) Shiga toxin is a secondary inhibitor of elongation factor 2-dependent reactions on eukaryotic ribosomes.
- 5) Following inactivation of ribosomes with Shiga toxin, a structural modification in protein or RNA components was not observed.
- 6) However, higher concentrations of Shiga toxin exhibited a ribonuclease activity with purified rRNA substrate.
- 7) The RNase activity of Shiga toxin was heat denatured at the temperature required to inactivate total protein synthesis inhibitory activity of the toxin.
- 8) The Shiga toxin-associated RNase activity differed in specificity from that of alpha-sarcin, a peptide toxin from the fungus Aspergillus.

## RECOMMENDATIONS

It would be helpful to know how eEF-1 and eEF-2 interact with 60S ribosomes during peptide elongation as it is our working hypothesis that Shiga toxin causes a structural change in the proximity of the ribosomal binding site for eEF proteins. Evidence has been presented that acidic r-proteins related to E. coli L7/L12 are required for eEF-1 and EF-2-dependent reactions on 60S ribosomes. More recently, others have shown that reticulocyte eEF-Tu, a 53,000 Da basic protein, contains a site which binds G-rich ribonucleotides such as 28S rRNA resulting in an enhanced GTPase activity of the eEF-Tu protein (25,26,27). Therefore, it would seem appropriate that efforts are directed towards a further elucidation of Shiga toxin interaction with rRNA species of 60S ribosomes.

A goal of these continued studies is to identify the molecular substrate of Shiga toxin which could be employed in a sensitive and selective detection assay for the toxin in biological samples. In addition, with such biochemical information one could also design substrates which may prove useful in toxin neutralization.

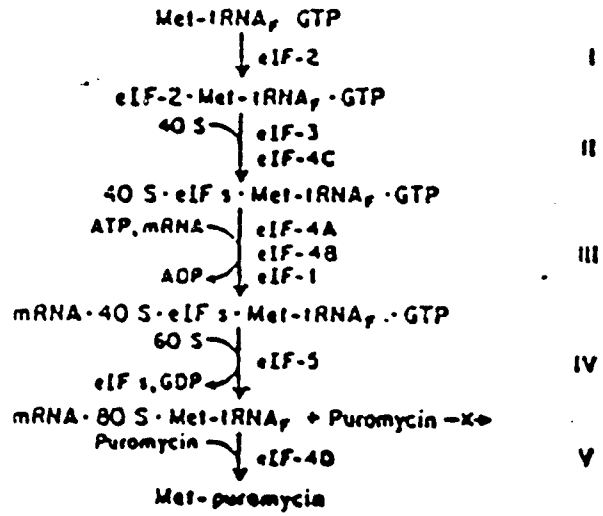


Fig. 1. Scheme for initiation of eukaryotic protein synthesis

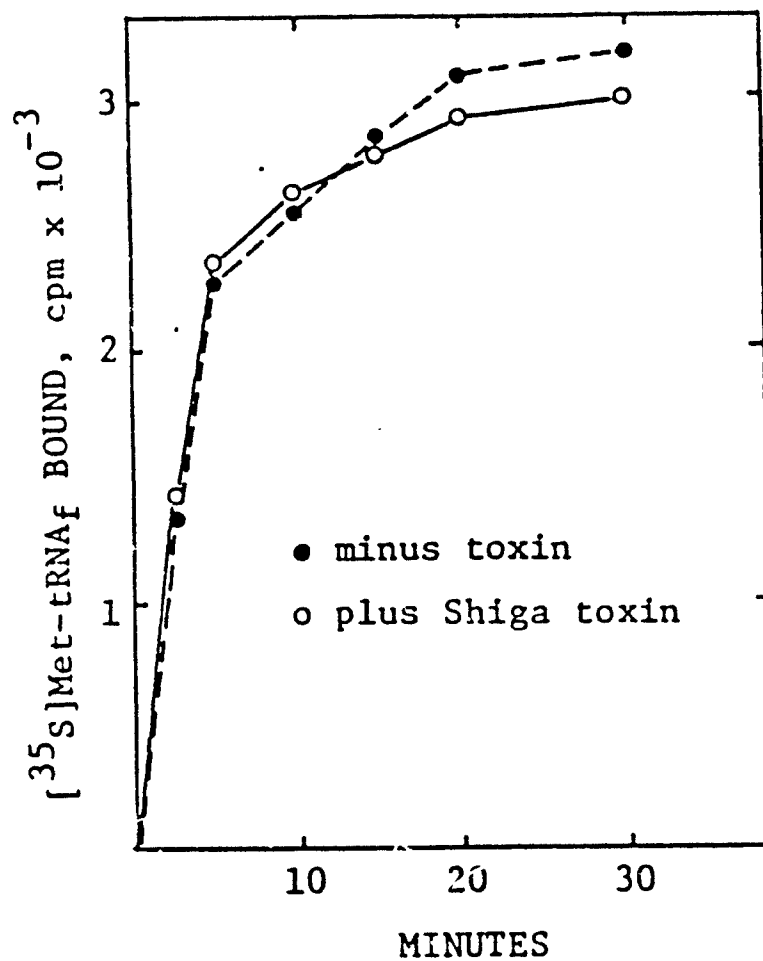


Fig. 2. Effect of Shiga toxin on formation of ternary initiation complex eIF-2 · [<sup>35</sup>S]Met-tRNA<sub>f</sub> · GTP. Measurement of this step was based on the concept that ternary initiation complex was retained on nitrocellulose filters whereas the individual components passed through. See Methods for experimental details. The results indicate Shiga toxin did not effect formation of the ternary initiation complex ie. step I in Fig. 1.

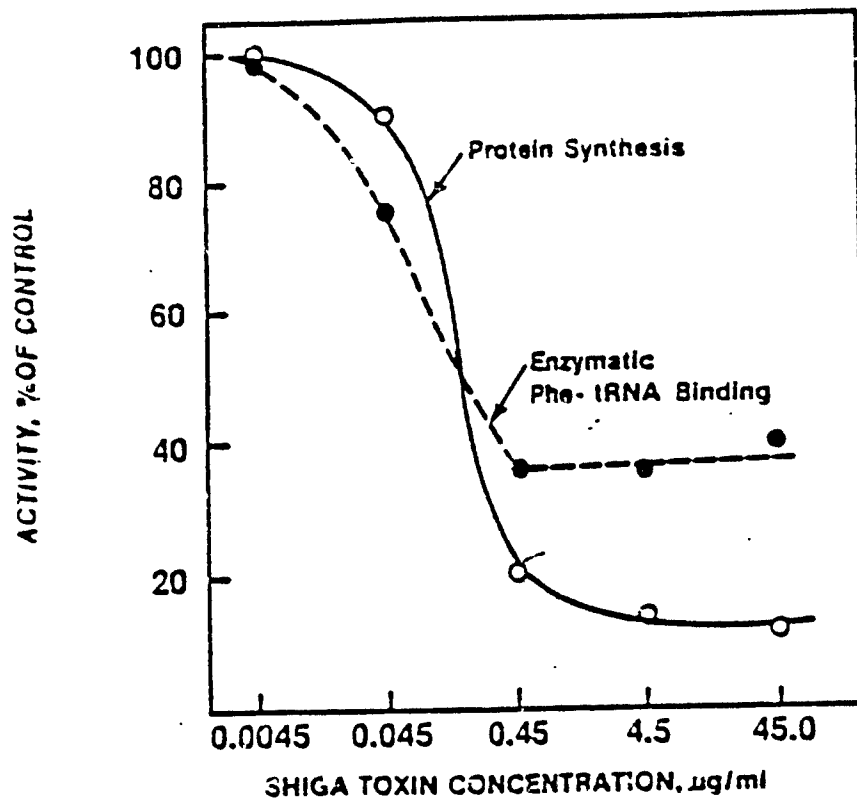


Fig. 3. Effect of Shiga toxin on total protein synthesis and phe-tRNA binding to reticulocyte ribosomes. A description of experimental procedures appears in the Methods section.

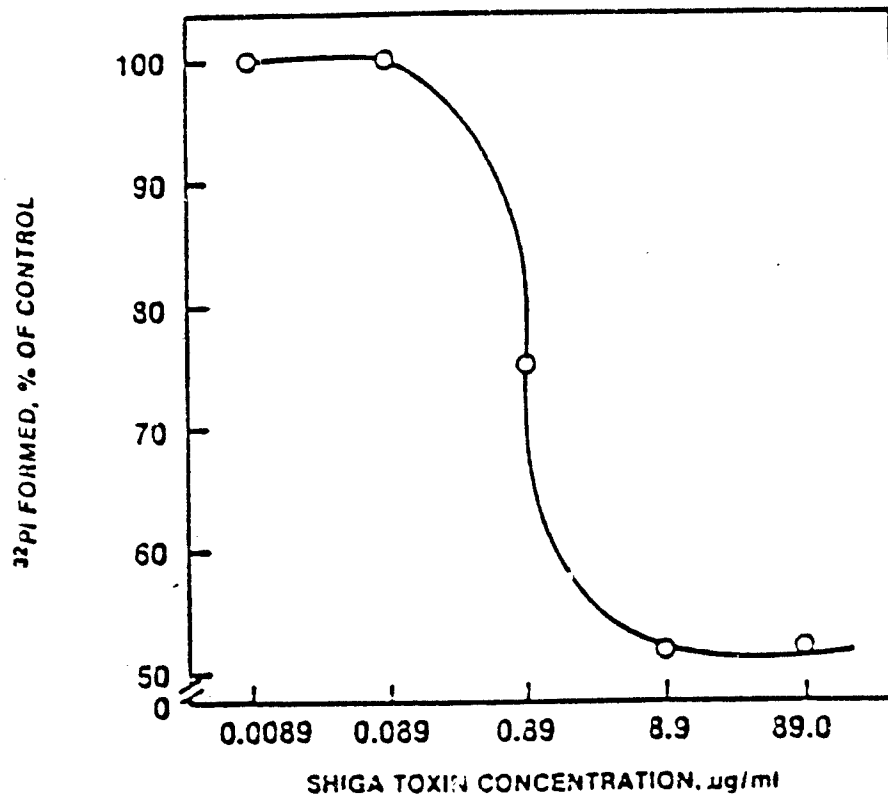


Fig. 4. Effect of Shiga toxin on EF1-dependent GTPase activity on reticulocyte ribosomes. Procedures of this assay are described in Methods.

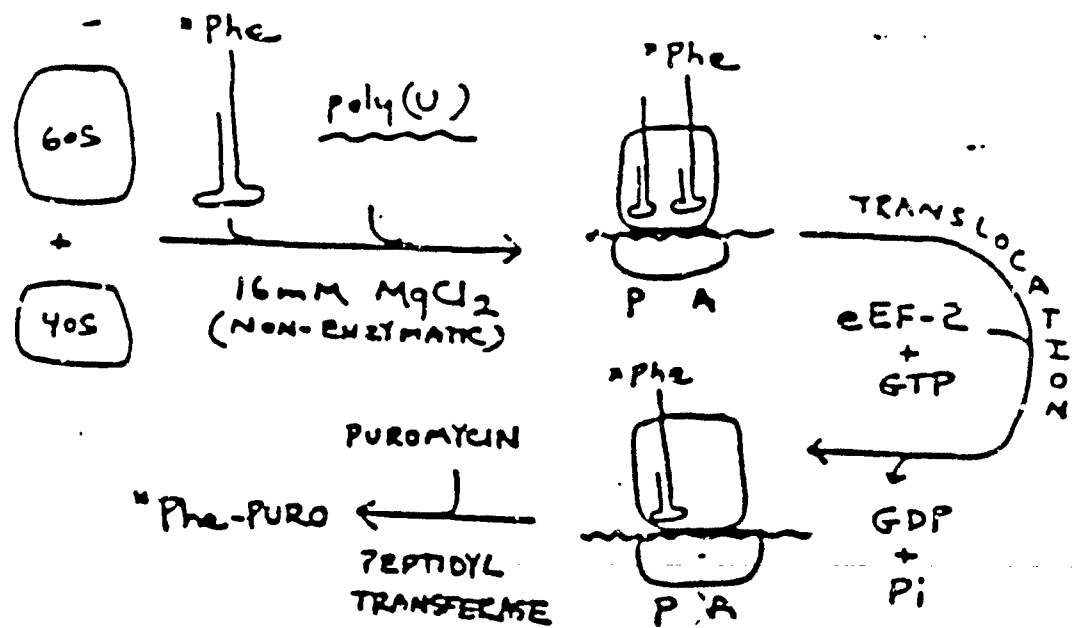


Fig. 5. The Coupled eEF-2 Translocation and Peptidyl Transferase Reaction. [<sup>3</sup>H]Phe-tRNA is non-enzymatically bound to purified ribosomes in a mRNA/dependent reaction. These ribosomes to which [<sup>3</sup>H]Phe-tRNA is bound into the " " site are reisolated and serve as substrate for the "coupled" reaction. The coupled reaction performed at 37°C in the presence of eEF-2, GTP and puromycin results in [<sup>3</sup>H]Phe-puromycin product formation.

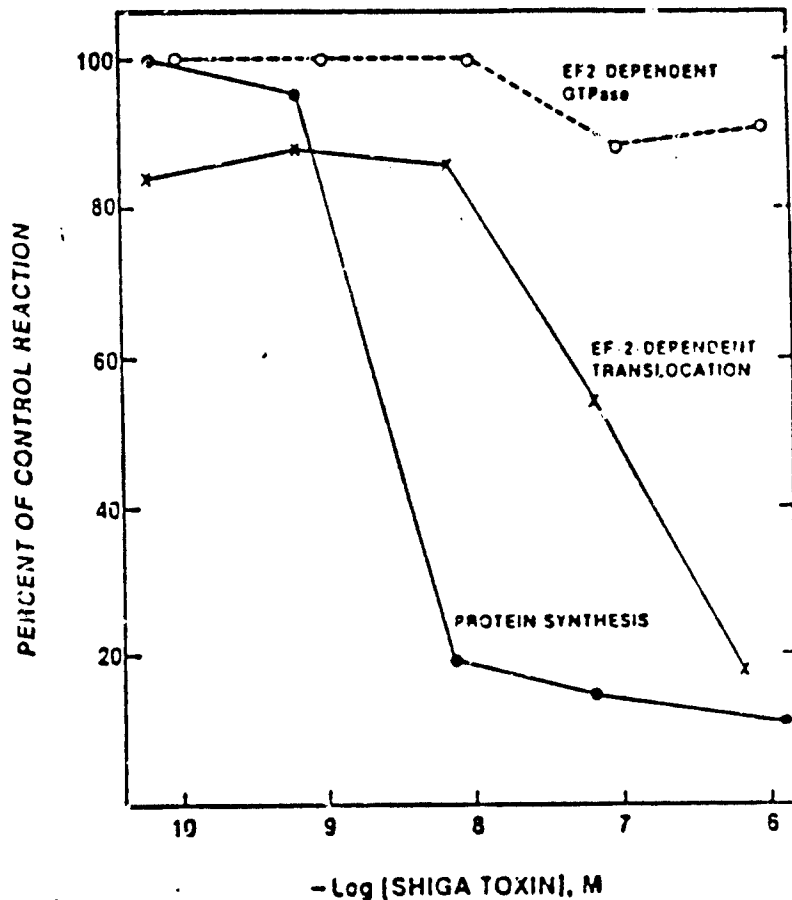


Fig. 6. The effect of Shiga toxin concentration on eEF-2 dependent translocation of aminoacyl-tRNA and eEF-2 GTPase activity. Enzymatic translocation of non-enzymatically bound [<sup>3</sup>H]Phe-tRNA on ribosomes (X—X) was carried out in the presence of eEF-2 protein and monitored with [<sup>3</sup>H]Phenylalanylpuromycin formation (see Methods). eEF-2 dependent GTPase activity (O—O) was measured in reactions containing ribosomes and [<sup>γ</sup>-<sup>32</sup>P]GTP as described in Methods. Total protein synthesis (●—●) was carried out in reticulocyte lysates as described, in Methods. Control 100% values for the three reactions were 2,340, 6,700, and 13,400 cpm, respectively.

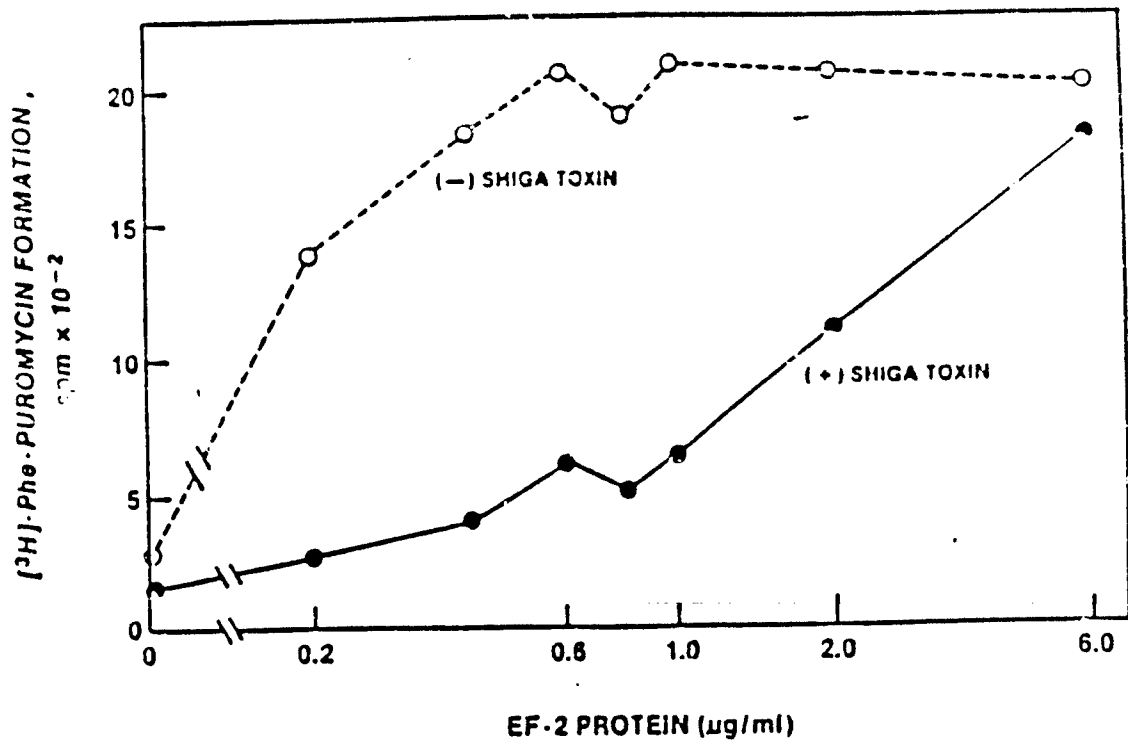


Fig. 7. The effect of eEF-2 protein concentration on Shiga toxin inhibition of aminoacyl-tRNA translocation on ribosomes. Reactions were performed in presence (●—●) or absence (O----O) of 1 μM Shiga toxin.

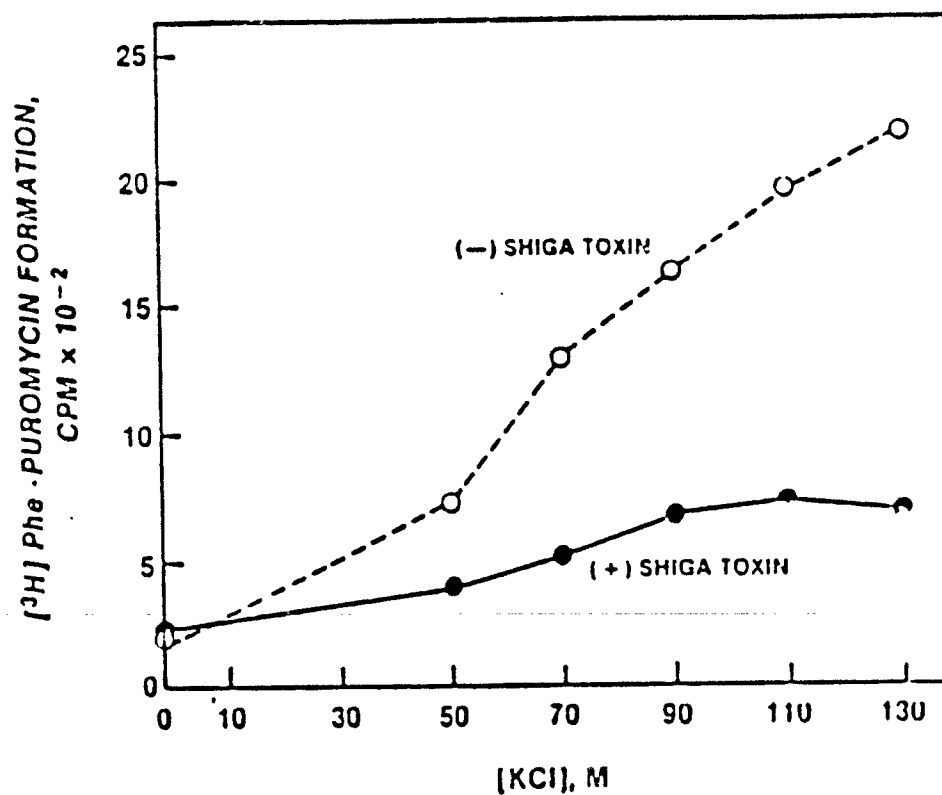


Fig. 8. The effect of KCl concentration on Shiga toxin inhibition of eEF-2 dependent of translocation aminoacyl-tRNA on ribosomes. Reactions mixtures were incubated in the presence (●—●) or absence (O----O) of 1  $\mu$ M Shiga toxin.

(-) Shiga toxin

(+) Shiga toxin

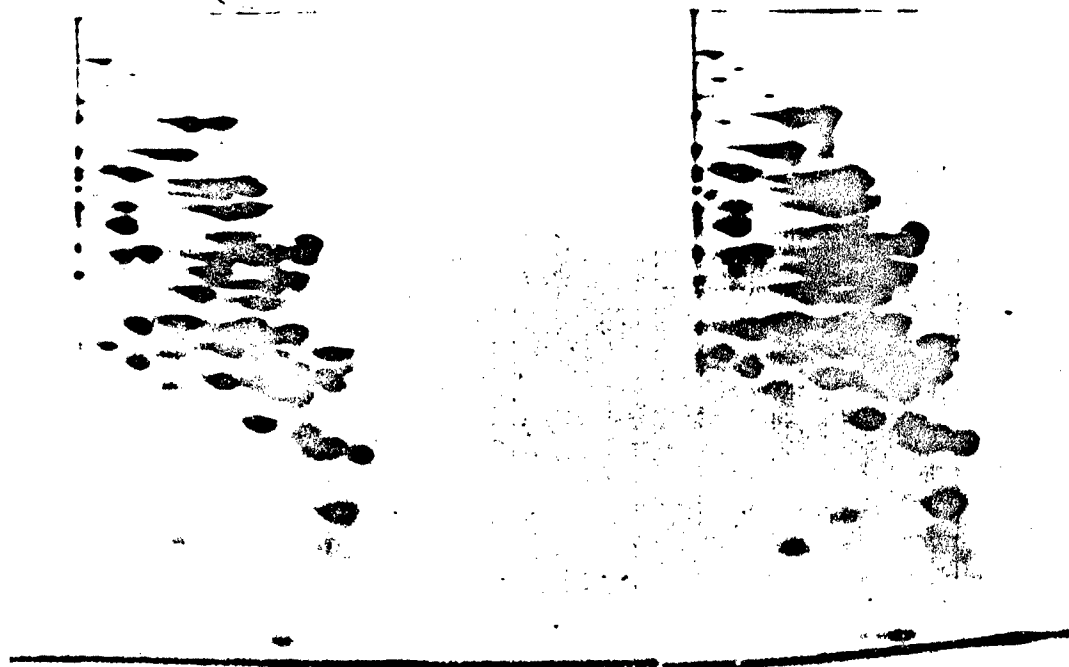


Fig. 9. 2-D Gel electrophoretic analysis of r-proteins extracted from toxin-treated ribosomes. Reticulocyte ribosomes were incubated with 10nM Shiga toxin at 37°C/1hr, then total r-protein was extracted with 1M acetic acid. Proteins from control or treated ribosomes were separated in the first dimension (left to right) at pH 8.6 in a 5% polyacrylamide tube gel. The second dimension separation was carried out by layering the tube gel on top of a 20% acrylamide slab gel and by electrophoresis (top to bottom) at pH 4.5. No difference was noted in the two samples.

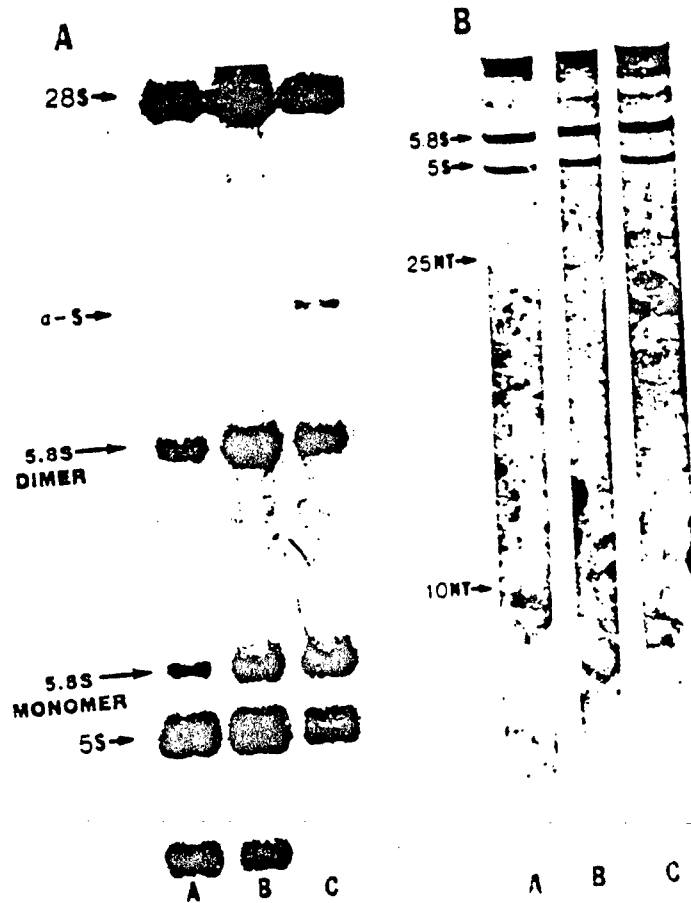


Fig. 10. A) Analysis of ribosomal subunit RNA from toxin-treated polysomes. Reticulocyte polysomes were treated with buffer (lane A) or with either Shiga toxin (lane B) or alpha-sarcin (lane C) and separated into subunits. Total RNA was extracted from 60S ribosomes,  $3'$ - $^{32}$ P end-labeled, and separated by electrophoresis on a 5% polyacrylamide gel. The alpha-sarcin RNA fragment is designated as  $\alpha$ -s. B) Analysis of 60S ribosomal subunit RNA from toxin-treated polysomes. Ribosomal RNA was isolated from 60S subunits of control (lane A), Shiga toxin (lane B) or alpha-sarcin (lane C) treated reticulocyte polysomes. Samples were separated by electrophoresis ( $1.5\mu\text{g}$  RNA/lane) on 20% polyacrylamide gels and detected by silver-staining. The markers, xylene cyanol and bromophenol blue, were included as determinants of 25 and 10 nucleotide fragments, respectively.

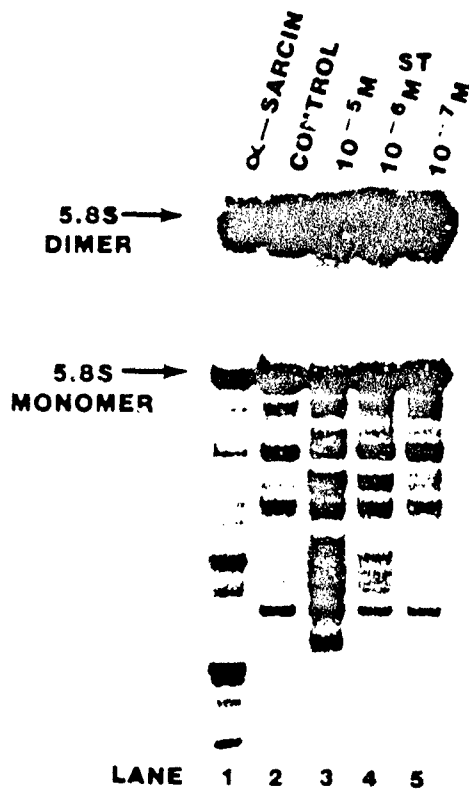


Fig. 11. RNase action of Shiga toxin on 5.8S rRNA substrate.  
 Different concentrations of Shiga toxin were incubated with purified 5.8S rRNA in the absence of magnesium. The RNA products were 3'-end labeled with <sup>32</sup>P, electrophoresed in a 10% acrylamide gel and detected by autoradiography. Alpha-sarcin treated RNA served as a positive control.

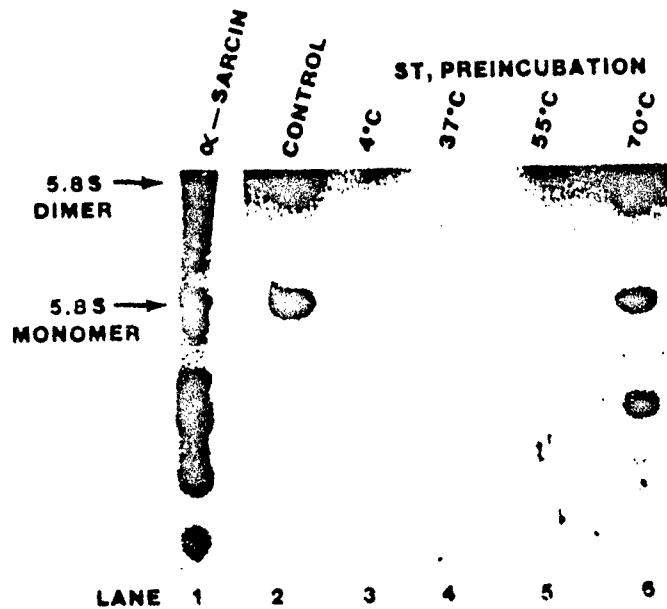


Fig. 12. Heat denaturation of Shiga toxin-associated RNase activity. Experimental procedures were as described in Fig. 11 and Methods. Where indicated, Shiga toxin ( $10^{-6}$  M) was preincubated for 10 min. at the stated temperatures prior to incubation with 5.8 rRNA.

TABLE 1: [<sup>35</sup>S]Methionylpuromycin formation in unfractionated reticulocyte lysate.

<u>Components</u>	<u>[<sup>35</sup>S], cpm</u>
minus lysate	315
complete, 4°C	265
complete, 37°C	4,315
complete, 37°C, Shiga toxin	3,885
complete, 37°C, HCR*	560

This assay demonstrates that Shiga toxin does not inhibit initiation of eukaryotic protein synthesis. [<sup>35</sup>S]Met-tRNA<sub>f</sub> was bound into the peptidyl site of 80S ribosomes in a mRNA-dependent manner, representing steps #1-4 in Fig. 1. By definition, aminoacyl-tRNA in the peptidyl-site is reactive with puromycin to yield \*Met-puromycin (Fig. 1, step 5). The \*Met-puromycin is extracted and measured as described in Methods.

\*HCR is hemin control repressor, a protein kinase activated in lysates incubated at 37°C without hemin. HCR phosphorylates (inactivates) initiation factor eIF-2. HCR was added to the assay, where indicated, at zero time as a positive control.

TABLE 2: Pentameric complex formation with purified components.

<u>Components</u>	<u>Filter-bound [<sup>35</sup>S], cpm</u>
minus 40S and Poly-AGU	90
minus 40S	88
minus Poly-AGU	130
minus ribosomal salt fraction	142
complete, incubation 0°C	94
complete, incubation 37°C	567
complete, 37°C, plus Shiga toxin	534

Shiga toxin did not affect pentameric initiation complex formation monitored in unfractionated reticulocyte lysates. The complex was formed among components: poly-AGU artificial mRNA, 40S ribosomes, [<sup>35</sup>S]Met-tRNA, GTP, and eIF-2 protein as shown in steps 1-3 of Fig. 1.

TABLE 3: Formation of pentameric initiation complex with natural messenger RNA.

<u>Assay Components</u>	<u>Filter-Bound [<sup>35</sup>S], cpm</u>
Complete	18,610 ± 1,070
Complete, incubated at 4°C	3,140 ± 286
Complete, + 1μg/ml Shiga toxin	14,770 ± 853
Complete + HCR*	5,360 ± 324

Shiga toxin did not inhibit formation of pentameric complex in the presence of globin mRNA. The complex, described in Table 2 was collected on nitrocellulose membrane filters or measured in sucrose gradients. Rationale for this assay procedure follows. Pentameric initiation complex was accumulated in reticulocyte lysate in response to added NaF and  $\beta$ ,  $\gamma$ -methylene GTP which inhibited the subsequent initiation step #4, Fig. 1, i.e., addition of 60S ribosomal subunits to the pentameric complex. If Shiga toxin activity inhibited one or more of the steps preceding step #4 one should see a reduced amount of pentameric complex formed. In this case, the sucrose gradients were employed for quantitative and qualitative analysis of [<sup>35</sup>S]Met-tRNA<sub>f</sub> binding into the pentameric initiation complex. Results from this assay were in full agreement with those obtained with the filter assay procedure. It was concluded that Shiga toxin did not inhibit steps #1 through #3 Fig. 1. The HCR\* protein was activated, in the absence of hemin, by preincubation (37°C/1hr) of 100,000 g supernatant of reticulocyte lysate. Results are presented as means ( $\pm$  S.E.M.) of four separate determinations.

TABLE 4: The effect of Shiga toxin on formation of [<sup>3</sup>H]GTP·eEF-2·ribosome complex.

<u>Additions</u>	<u>[<sup>3</sup>H]GTP·eEF-2-Ribosome Complex Formation</u>	
	<u>cpm</u>	<u>% Control</u>
Control	5,312	100
Shiga toxin, 0.1 nM	5,283	99
Shiga toxin, 10 nM	5,205	98
Shiga toxin, 1 μM	5,011	94
Phytolaccin, 0.1 μM	720	13

Ternary complex formation conducted in the presence of purified eEF-2 protein and 0.5 M KCl-washed ribosomes, was monitored by collection on nitrocellulose filters. Toxins were added to reactions to yield the final concentrations indicated. Data are presented as a percent incorporation compared to complete reaction mixtures performed in the absence of inhibitors. Shiga toxin did not inhibit formation of this complex.

TABLE 5: The effect of Shiga toxin on eEF-2-dependent translocation and peptidyl transferase reactions.

<u>1st Incubation</u>	<u>2nd Incubation</u>	<u>[<sup>3</sup>H]Phe-puromycin formed, % of control</u>
----	---	100
Shiga toxin, 1 $\mu$ M	---	45
----	Shiga toxin, 1 $\mu$ M	100
Alpha Sarcin, 1 $\mu$ M	---	65
----	Alpha Sarcin, 1 $\mu$ M	87
Phytolaccin, 1 $\mu$ M	---	23
----	Phytolaccin, 1 $\mu$ M	92
Cycloheximide, 1 mM	---	56
----	Cycloheximide, 1 mM	98

Translocation of non-enzymatically bound [<sup>3</sup>H]Phe-tRNA on reticulocyte ribosomes was performed as described in Methods. Translocation of [<sup>3</sup>H]Phe-tRNA from the A to P-site on ribosomes was carried out during the first incubation in the presence of eEF-2 protein and GTP. The reaction mixture was then cooled to 4°C, puromycin added and [<sup>3</sup>H]Phe-puromycin formation allowed to proceed in the second incubation. Inhibitors were added either in the first or second incubation as indicated below. Control (100%) formation of [<sup>3</sup>H]Phe-puromycin was 2236 cpm. Shiga toxin inhibited the eEF-2 dependent translocation, but was without effect on peptidyl transferase activity.

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