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MODE OF ACTION OF SHIGELLA TOXIN: EFFECTS ON
RIBOSOME STRUCTURE AND FUNCTION

Annual Report

Tom G. Obrig, Ph.D.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) SUMMARY → 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 ribosome 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		

20. (Summary continued)

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 Shigella species. In this contract period we have further delineated how 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 have concluded 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 period 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.



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FOREWORD

In conducting the research described in this report, the investigator 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 (DHEW Publication No. NIH-78-23, Revised, 1978).

BODY OF THE REPORT

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 suggest 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 (11,14,30). 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. Recent data from our laboratory indicates that the toxin may be a ribonuclease (32,37).

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.

Approach to the Problem

a. GENERAL INFORMATION

It seems very possible that Shiga toxin could share many of the features of the plant-derived phytolectin toxin as an inhibitor of protein biosynthesis (24). Our research plan is designed to examine the action of Shiga toxin in the test systems we have successfully employed in the study of plant toxin mode of action. These investigations were designed, in part, because the Principal Investigator has had extensive experience in preparation and use of protein synthesis assay systems which would be useful in carrying out the Shiga toxin study (22-25).

The primary objective of this project is to describe, in biochemical terms, how Shiga toxin inhibits protein biosynthesis in mammalian cells. To achieve this goal, when this project was initiated we set out to examine two aspects of protein synthesis in reticulocyte (rabbit) cell-free protein synthesis systems. First, we determined what steps of protein synthesis (i.e., initiation or elongation) were affected by Shiga toxin. Indeed, we established that the toxin did not inhibit initiation, but was a potent inhibitor of at least one step of peptide elongation, elongation factor 1 catalyzed aminoacyl-tRNA binding to ribosomes (31,35). The present goal is to examine the effect of Shiga toxin on elongation factor 2-dependent translocation of peptidyl-tRNA and also peptidyl transferase activity. Second, we wanted to determine how Shiga toxin inactivated mammalian ribosomes. More exactly, as Shiga toxin is known to specifically inactivate the large ribosomal subunit, a goal of this research is to determine how Shiga toxin modifies the structural integrity of the ribosome. Components of the large ribosomal subunit including its 47 proteins and 3rRNAs was monitored for changes resulting from Shiga toxin action. No changes were observed (33). The following is an abbreviated description of methodology used in the continued study of Shiga toxin mode of action as an inhibitor of protein biosynthesis in defined assay systems.

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^f - 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

b. METHODS

(1) 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 ug 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 ug 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.

(2) 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 MgCl₂) 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 decxycholate, 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.

(3) 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.

(4) [³H] Phe-tRNA Synthesis - Aminoacylation of yeast tRNA with [³H]Phe was carried out in 4.0 ml reaction containing: 100 mM Tris-Cl, pH 7.4, 10 mM Mg(OAc)₂, 10 mM dithiothreitol, 2 mM ATP, 2.6 mg creatine phosphate, 0.1 mg creatine phosphokinase, 200 ug of reticulocyte ribosomal 0.5 M KCl wash protein, 5 mg tRNA, and 100 ug [³H]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. [3H]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 [3H]Phe-tRNA precipitated by addition of 2.5 vol of 95% ethanol at -20°C/2 h. The [3H]Phe-tRNA pellet was washed with 95% ethanol and then with ethyl ether to remove residual phenol.

(5) eEF-1 and eEF-2 GTPase Assay - The eEF-2 GTPase reaction (31) mixture was performed in a total volume of 50 ul containing, in order of addition: 25 mM Tris-Cl, pH 7.4, 100 mM KCl, 5 mM Mg(OAc)₂, 2.5 mM dithiothreitol, Shiga toxin as indicated, 1.0 A260 units DOC-KCl washed ribosomes, 0.2 ug eEF-2, and 5 uM [-32P]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 H₂SO₄, 0.5 ml 1 mM potassium phosphate, pH 6.8, and 0.25 ml of 5% (W/V) ammonium molybdate in 4 N H₂SO₄. 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.

(6) eEF-2 Translocation Assay - [3H]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 MgCl₂, 5 mM dithiothreitol, 10 mg poly(U), 620 A260 units of DOC-KCl washed ribosomes, and 3 nmol of [3H]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 MgCl₂ 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 MgCl₂, 1 mM 2-mercaptoethanol and 10% glycerol at 125 A260 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 MgCl₂, 5 mM dithiothreitol, 3.0 A260 units DOC-KCl washed ribosomes with [3H]Phe-tRNA (5,000 cpm) non-enzymatically bound as described above, activated Shiga toxin as indicated, 0.5 ug 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. [3H]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.

(7) [eEF-2.GTP.80S ribosome] Complex Formation The 100 ul reaction mixture contained the following components listed in order of addition: 25 mM Tris-Cl, pH 7.4, 110 mM K(OAc), 5 mM Mg(OAc)₂, 2.5 mM dithiothreitol, 1.0 A260 unit DOC-KCl washed ribosomes (21 pmol), 0.4 ug eEF-2 protein, Shiga toxin as indicated, and 1 uM [3H]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 BA85, 0.45 u 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.

(8) 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°/10min. Concentrations of toxins are noted in each figure. Following incubation, rRNA was 3' end-labeled at 4°C/12hr. with [32P-5']pCp, S.A.=2500 Ci/mmol, and T4 ligase as described by Peattie (38). The sample was precipitated with 70% ethanol and dissolved in electrophoresis sample buffer consisting of : 8M urea, 20mM Tris-HCl, pH 7.4, 1mM Na₂-EDTA, 0.05% xylene cyanol and 0.05% bromophenol blue. Samples (5ul) 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

a. Elongation of Protein Synthesis: Effect of Shiga Toxin on eEF-2 Reactions

- 1) Shiga Toxin Inhibits the Coupled eEF-2 Translocation and Peptidyl Transferase Reaction. This "coupled" reaction is presented schematically in Fig. 1. When this reaction was carried out in the presence of Shiga toxin, formation of [3H]Phe-puromycin was inhibited in a dose-dependent manner (Fig. 2). 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. 2). Data gathered in the prior contract period revealed that eEF-1 dependent Phe-tRNA binding to ribosomes and overall protein synthesis were equally sensitive to Shiga toxin. Thus, the toxin is a more potent inhibitor of these two systems than of eEF-2 dependent translocation.

We then 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. 3). Translocation reactions were saturated by 0.6 ug eEF-2 protein in the absence of toxin, but in the presence of Shiga toxin required 6.0 ug of eEF-2 protein to reach plateau level (Fig. 3). 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 130 mM range had only a slight effect on Shiga toxin's ability to inhibit [3H]Phe-puromycin formation (Fig. 4). 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.

- 2) 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.1). [3H]Phe-tRNA is bound non-enzymatically into the ribosomal A-site. The first step involves the eEF-2-dependent movement of [3H]Phe-tRNA on ribosomes from the A- to P- site. The second incubation involves reaction by peptidyl transferase of the [3H]Phe-tRNA located in the P-site with puromycin to yield [3H]Phe-puromycin. When 1 uM Shiga toxin was present during the peptidyl transferase step (i.e. 2nd incubation), no effect on that activity was detected (Table 1).

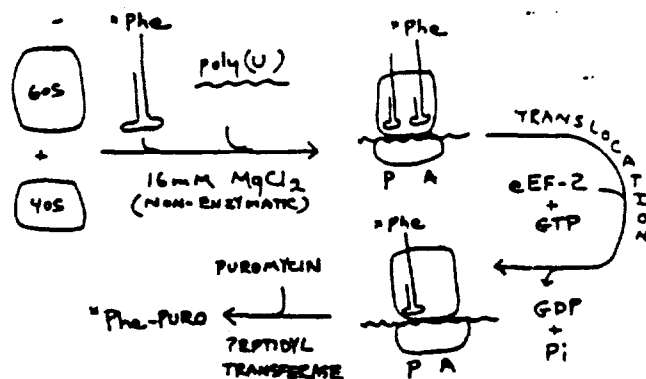


Fig. 1 The Coupled eEF-2 Translocation and Peptidyl Transferase Reaction
 $[^3\text{H}]\text{Phe-tRNA}$ is non-enzymatically bound to purified ribosomes in a mRNA-dependent reaction. These ribosomes to which $[^3\text{H}]\text{Phe-tRNA}$ is bound into the "A" site are reisolated and serve as substrate for the "coupled" reaction. The coupled reaction performed at 37°C in the presence of eEF-2, GTP and puromycin results in $[^3\text{H}]\text{Phe-puromycin}$ product formation.

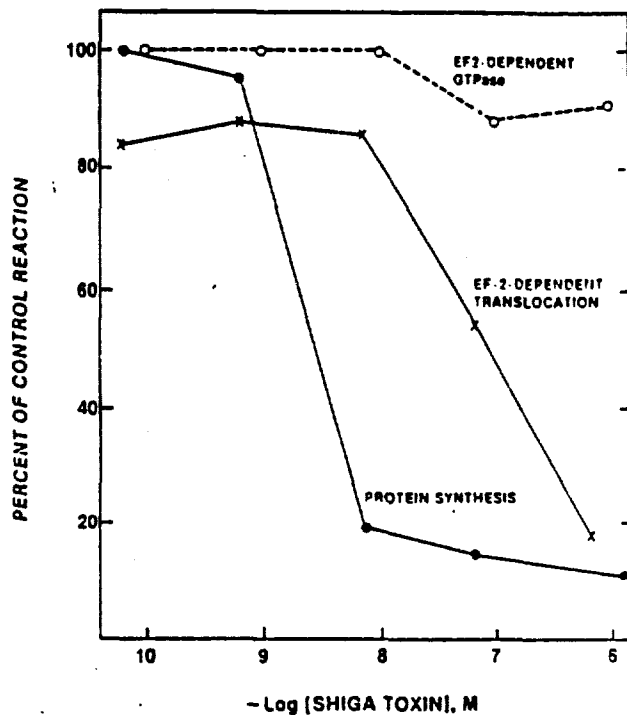


Fig. 2. The effect of Shiga toxin concentration on eEF-2 dependent translocation of aminoacyl-tRNA and eEF-2 GTPase activity.

Enzymatic translocation of non-enzymatically bound $[^3\text{H}]\text{Phe-tRNA}$ on ribosomes (X—X) was carried out in the presence of eEF-2 protein and monitored with $[^3\text{H}]\text{Phenylalanylpuromycin}$ formation (see Methods). eEF-2 dependent GTPase activity (O—O) was measured in reactions containing ribosomes and $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ as described in Methods. Total protein synthesis (●—●) was carried out in reticulocyte lysates as described, Control 100% values for the three reactions were 2,340, 6,700, and 13,400 cpm, respectively.

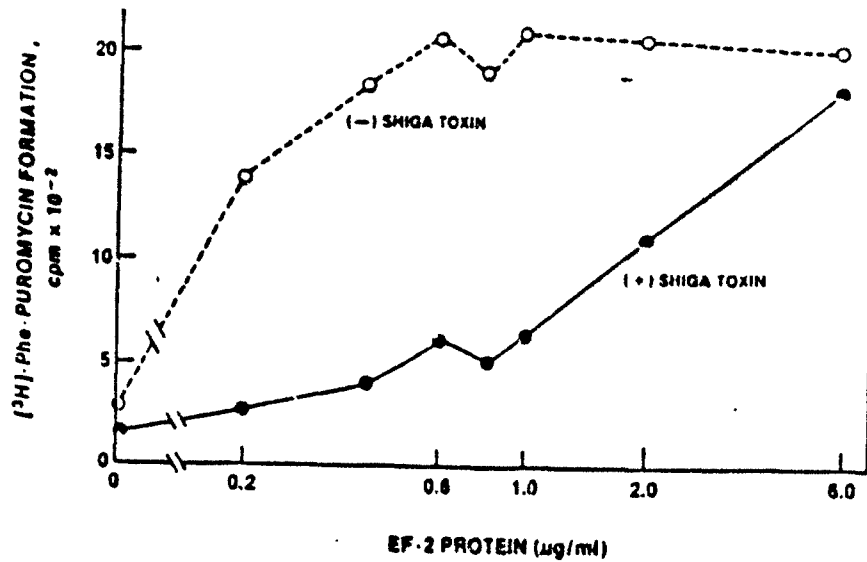


Fig. 3. The effect of eEF-2 protein concentration on Shiga toxin inhibition of aminoacyl-tRNA translocation on ribosomes.

Reactions were performed in the presence (●—●) or absence (○—○) of 1 μM Shiga toxin as described in Fig. 1.

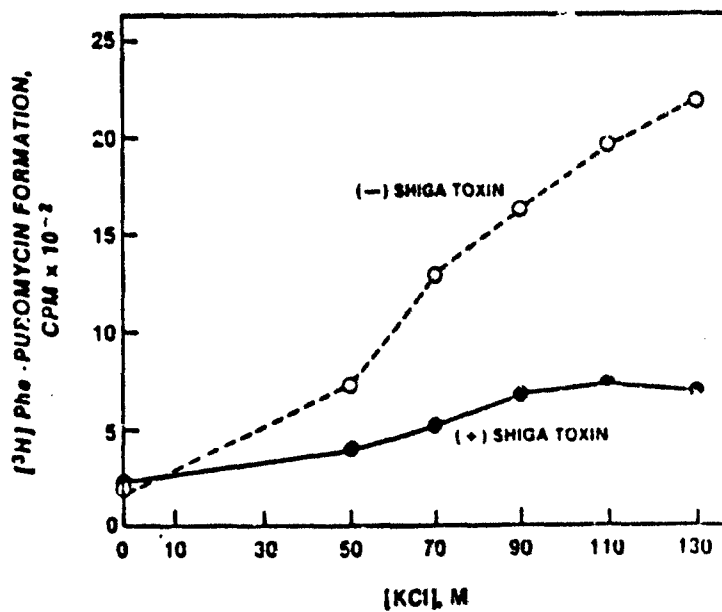


Fig. 4. The effect of KCl concentration on Shiga toxin inhibition of eEF-2 dependent of translocation aminoacyl-tRNA on ribosomes.

Reactions mixtures were incubated in the presence (●—●) or absence (○—○) of 1 μM Shiga toxin as described in Fig. 1.

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

Translocation of non-enzymatically bound [³H]Phe-tRNA on reticulocyte ribosomes was performed as described in Methods. Translocation of [³H]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 [³H]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 [³H]Phe-puromycin was 2236 c.p.m.

1st Incubation	2nd Incubation	[³ H]Phe-puromycin formed, % of control
—	—	100
Shiga toxin, 1 μM	—	45
—	Shiga toxin, 1 μM	100
Alpha sarcin, 1 μM	—	65
—	Alpha sarcin, 1 μM	87
Phytolaccin, 1 μM	—	23
—	Phytolaccin, 1 μM	92
Cycloheximide, 1 μM	—	56
—	Cycloheximide, 1 μM	98

TABLE II The effect of Shiga toxin on formation of [³H]GTP-eEF-2-ribosome complex.

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 as described in Methods. 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.

<u>Additions</u>	<u>[³H]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

- 3) Shiga Toxin Inhibits the Isolated Translocation Reaction
Shiga toxin (1 μ M) added during the translocation step reduced [³H]Phe-puromycin formation to 45% of control values. Puromycin was absent from the reaction, thus only translocation was being measured. Subsequent to this incubation, puromycin was added and the second ie. 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 1).
- 4) Shiga Toxin Does Not Inhibit eEF-2 GTPase The effect of Shiga toxin or eEF-2 dependent GTPase activity was examined over a concentration range of 0.1 nM to 1 μ M toxin (Fig. 2). 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 μ 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.
- 5) 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 μ M did not affect complex formation (Table 2). Thus, Shiga toxin appears to differ from other ribosome-inactivating toxins such as phytolectin and abrin which are strong inhibitors of 3° complex formation.

b. Shiga Toxin Exhibits Ribonuclease Activity on Free rRNA Substrate

A study 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 [³²P-5']_pCp and T4 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. 5). It was also determined that this activity was time-dependent as incubation of 10⁻⁶M Shiga toxin with the 5.8S rRNA resulted in extensive degradation of the RNA within 30min. (Fig. 6, lanes 3-5). The RNA fragment patterns differed for Shiga toxin and alpha-sarcin incubation reactions (Fig. 5, 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. 6). 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.

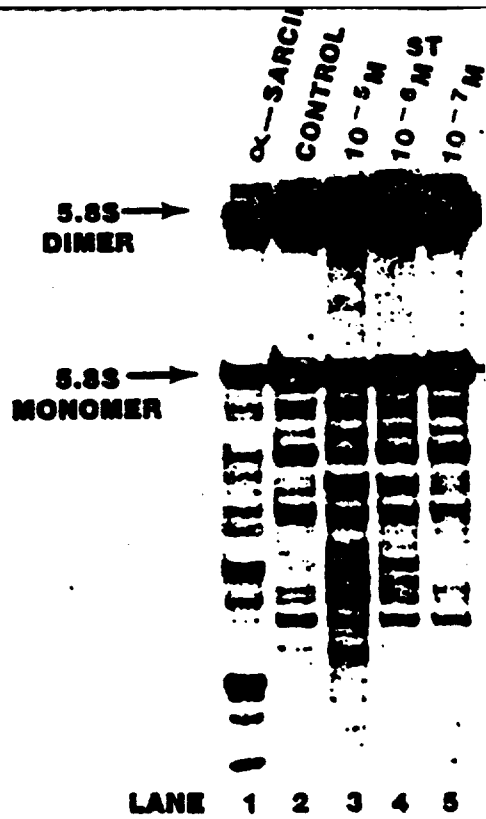


Fig. 5. RNase action of Shiga toxin (ST) on 5.8S rRNA substrate. Experimental procedures were as described.

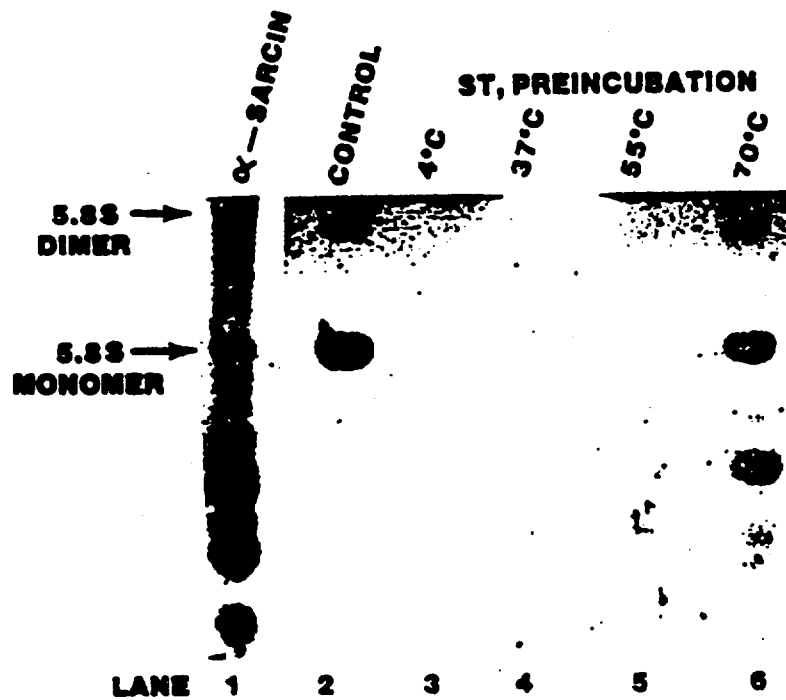


Figure 6. Heat denaturation of Shiga toxin (ST) RNase activity. Experimental procedures were as described. Where indicated ST ($10^{-6}M$) was preincubated 10min. at the stated temperatures prior to incubation with 5.8S rRNA.

a. Summary of Shiga Toxin Action on Peptide Elongation Steps of Protein Synthesis

In this investigation, we examined the effect of Shiga toxin on defined peptide elongation reactions of eukaryotic protein synthesis. Our present results coupled to those from the previous contract periods demonstrate that the primary functional lesion induced by Shiga toxin is a direct inhibition of eEF-1 dependent aminoacyl-tRNA binding to ribosomes. The results show that Shiga toxin inhibits both enzymatic [³H]Phe-tRNA binding to ribosomes and eEF-1 dependent GTPase activity at toxin concentrations similar to those required for inhibition of overall protein synthesis in reticulocyte lysates and that the inhibition of [³H]Phe-tRNA binding was not overcome by increased concentrations of eEF-1 protein. This year we have shown that 1) peptidyl transferase activity was not affected and 2) in contrast to eEF-1-dependent aminoacyl-tRNA binding, a more than 20 fold higher toxin concentration was required to inhibit the eEF-2-dependent translocation process by 50%. Increased concentrations of eEF-2 restored translocation activity to full levels. Moreover, effects of Shiga toxin on both eEF-2 dependent GTPase and [³H]GTP.eEF-2. ribosome complex formation are negligible.

These data, combined with previous findings, begin to describe a detailed picture of the effect of Shiga toxin on ribosome function. We have recently demonstrated that the peptide initiation process is unaffected by Shiga toxin (30). Similarly, Shiga toxin does not inhibit aminoacylation of tRNA (13) or peptidyl transferase reactions on eukaryotic ribosomes (15). Studies conducted with crude reticulocyte lysate have provided indirect evidence that Shiga toxin exhibits primary effect on aminoacyl-tRNA binding to ribosomes (30).

Shiga toxin resembles other ribosome-inactivating catalytic protein toxins, such as ricin, abrin, phytolectin (previously termed PAP) and alpha sarcin. All of these toxins inhibit protein synthesis as a result of direct action on the 60S ribosomal subunit (19). However, our results also reveal a major difference between Shiga toxin and the other toxins. eEF-1 dependent binding of aminoacyl-tRNA to ribosomes appears to be the primary reaction inhibited by Shiga toxin. Although the other toxins may inhibit this step, they appear to preferentially affect eEF-2 dependent translocation reactions. These findings suggest that ribosomes inactivated by Shiga toxin have a greatly reduced affinity for eEF-1 protein whereas ribosomes inactivated by ricin or phytolectin exhibit a much reduced affinity for eEF-2 protein. This would help to explain why these specifically inhibited reactions are not easily reversed upon addition of excess corresponding elongation factor.

It is still possible that a large excess of eEF-1 protein in whole cells could prevent Shiga toxin inhibition of aminoacyl-tRNA binding to ribosomes. However, our calculations indicate that conditions in the [³H]Phe-tRNA binding assay of the present study closely match the relative amounts of eEF-1 protein and ribosomes found in crude reticulocyte lysate (39). In both cases, eEF-1 protein is present in a 25-fold molar excess compared to ribosomes. Therefore, we must conclude that Shiga toxin would indeed be a potent inhibitor of [³H]Phe-tRNA binding to ribosomes in whole cells.

Shiga toxin is a less potent inhibitor of the eEF-2-dependent translocation reaction. Conditions for this defined reaction in the present study actually favored toxin inhibition of translocation as eEF-2

protein was limiting. The molar ratio of eEF-2 to ribosomes was 1:3 in the defined reaction as compared to a 1:1 ratio normally present in reticulocyte lysates (39). Thus, our data suggest that Shiga toxin would have a very limited inhibitory effect on eEF-2-dependent reactions in reticulocyte lysates and whole cells. Indeed, indirect measurements of eEF-2 reactions in such lysates have been carried out and confirm this concept (30).

b. Shiga Toxin Ribonuclease Activity

Having established directly that Shiga toxin causes a specific functional lesion in peptide elongation, we also have need to answer how Shiga toxin inactivates 60S ribosomes. It is unlikely that Shiga or other similar toxins must bind firmly and stoichiometrically to ribosomes for continued inactivation. To the contrary, all data available indicate these toxins inactivate 60S ribosomes catalytically. To date, we and others have failed to detect a change in any of the 47 r-proteins or 3 rRNA species following toxin-inactivation of 60S ribosomes. The two exceptions are alpha-sarcin and colicin E3 which were shown to be specific ribonucleases of 28S and 16S rRNAs of intact 60S and 30S ribosomes, respectively (40,41). More recently we have observed that Shiga toxin as well as ricin and phytolectin preparations possess a RNase activity using free 5.8S rRNA as a substrate (32). Shiga toxin appears to prefer hydrolysis of single-stranded regions located in hairpin loops of 5.8S rRNA. Other data indicate that the temperature at which Shiga toxin heat denatures there is a concomitant loss of both protein synthesis inhibitory and RNase activities (32). Further characterization of this RNase activity may help to reveal the specific structural lesion associated with Shiga toxin inactivation of the 60S subunit.

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 aminoacyl-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.

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 (42,43). 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 (39,44,45). 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.

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