AN ABSTRACT OF THE THESIS OF

Martin L. Pollock for the degree of Master of Science in Biochemistry and Biophysics presented on August 19, 1986.
Title: BIOCHEMICAL STUDIES ON THE MOSQUITOCIDAL DELTA ENDOTOXIN OF *Bacillus thuringiensis* subsp. *israelensis*.

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George S. Beaudreau

The cytolytic properties of the mosquitocidal 25 Kd protein delta-endotoxin of *Bacillus thuringiensis* subsp. *israelensis* (Bti) was studied using the human erythrocyte as a target cell. Both a fixed-time and a kinetic hemolytic bioassay were developed in order to screen a variety of treatments for their influence on the toxin-erythrocyte interaction. Upon addition of 4 to 8 nM toxin to 2.5 X 10^7 cells/ml, at 37°C, after an initial 10 minute lag, hemolysis began and reached a maximum value in 60 minutes. The extent of hemolysis was independent of cell concentration over an 18.4-fold range. Toxin was equally active from pH 6 to 9.5, but hemolysis was reduced at pH 5 and 10. Lowering the pH from 10 to 9 restored about 40% of the activity. Hemolysis was temperature dependent; lowering the temperature below 37°C caused an increase in the reaction lag time and a reduction in total hemolysis in 60 minutes. Raising the temperature to 47°C did not alter the extent or kinetics of hemolysis as compared to 37°C.

Potassium release preceded hemoglobin release, suggesting that if a pore was formed, it was not stable for a long period of time. Hemoglobin release followed a single phase first-order efflux, but potassium efflux appeared to be multiphasic. 40 mM calcium inhibited hemolysis, regardless of the time that it was added during the assay. Magnesium also inhibited hemolysis but it was 6 times

less potent than calcium. However, very low concentrations of magnesium stimulated lysis. When toxin was removed in the early stages of the assay, a reduction of hemolysis was seen, which suggested that the toxin was binding weakly to the membrane.

From the lack of the effect of DTT, EDTA, and EGTA on hemolysis, it appeared that the toxin-erythrocyte interaction did not require intact disulfide bonds or divalent metals. Lack of hemolysis inhibition by BSA and sucrose suggested the lysis was not due to a colloid osmotic effect. Substitution of KCl for NaCl did not alter lysis kinetics. Addition of ATP to the assay did not alter hemolysis, suggesting that positive charges on the toxin or erythrocyte were not required at pH 7.4. Addition of a purified monoclonal antibody to the Bti toxin did not inhibit lysis. Bti toxin was not interacting with a sialic acid-containing receptor because neuraminidase-treated erythrocytes failed to alter the extent of lysis.

The target for the Bti toxin was neither an ion channel nor the Na^+/K^+ ATPase because specific ion channel and pump antagonists failed to inhibit lysis. This finding was also supported through the use of ATP-depleted cells, which did not affect lysis, suggesting that cell lysis was not dependent on an ATP-requiring membrane pump.

Biotinylated toxin was biologically inactive on erythrocytes, suggesting that lysines were essential for activity. Toxin labelled with FITC demonstrated some binding to lysed cells, but not to intact cells. Toxin was also able to lyse nucleated erythrocytes, (chicken cells), but they were less sensitive than human cells.

The Bti toxin interaction with *Aedes albopictus* insect cells was sensitive to heat at 50°C for 50 minutes. NaCl protected this inactivation at 50°, but not at 60°C. The heat-denatured toxin may have been aggregated, as it failed to migrate into a native polyacrylamide gel and it bound 2.5 times more Coomassie blue dye.

Protein structure changed with heat treatment, as the fluorescence spectra showed a decrease in peak height but no change in the wavelength. The toxin-erythrocyte interaction was also heat-sensitive, as 50°C for 60 minutes inactivated the toxin by 40%; no renaturation was seen for up to 4 hours after heat removal.

The Bti toxin interaction with insect cells was also sensitive to 6 M urea treatment as the physical properties of the protein changed, causing a loss of fluorescence peak height, a red wavelength shift, and loss of biologic activity. Upon urea removal, there was a partial restoration of the fluorescence parameters, but not of the biological activity. Excess dye-binding and lack of gel migration suggested, like heat denaturation, that the toxin was forming an aggregate or undergoing some other type of structural change.

Bti toxin was examined for phospholipase C activity which proved to be negative. A tryptophan content analysis of the Bti toxin showed three tryptophans per toxin molecule. From all of these studies and reports of other investigators, a tentative model for the mode of action of the Bti toxin-erythrocyte lysis process is suggested, which provides a basis for additional studies.

BIOCHEMICAL STUDIES ON THE MOSQUITOCIDAL DELTA ENDOTOXIN OF *Bacillus thuringiensis* subsp. *israelensis*

by

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DEDICATION

I dedicate this thesis to my beloved father, Irving (1922-1986), whose humble, compassionate, and loving spirit are deeply missed, and who will be an eternal source of hope and admiration for me. I also dedicate this thesis to my wonderful mother, Selma (1918-), whose love for me is unceasing, and to whom I am looking forward to a new and joyful relationship in the coming years.

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INTRODUCTION

The different subspecies of *Bacillus thuringiensis* are of agricultural importance as they have found use as biological insecticides against a wide variety of insects that infect various crops (Table 1, Luthy and Ebersold 1981b). *B. thuringiensis* microorganisms differ from other *Bacillus* species, such as *B. cereus* and *B. anthrax*, in that upon sporulation, a parasporal crystal or inclusion is formed (Figure 1). The crystal is a protein matrix held together by disulfide and hydrophobic bonds (Nickerson 1980). Upon ingestion of the crystal by an insect, it is solubilized in the alkaline insect midgut and a protein toxin is released which kills the insect (Bulla et al. 1980). The protein toxin is called the "delta"-endotoxin to distinguish it from other *Bacillus thuringiensis* non-occluded toxins called the alpha and beta toxins, which have a non-specific toxicity to many orders of insects and other organisms. (Luthy and Ebersold 1981, Lecadet and de Barjac 1981).

Table 2 lists the different subspecies of *Bacillus thuringiensis* that are known which have shown entomopathogenic activity. One of these subspecies, *israelensis* (Bti, serotype 14), discovered in 1977 by Goldberg, is of great medical and public health importance because it is lethal for the larvae of certain dipteran insects such as mosquitoes and blackflies. Mosquitoes are the vectors of yellow fever (*Aedes*), malaria (*Anopheles*) and filiarasis (*Culex*) (Goldberg and Margalit 1977). The World Health Organization regards Bti as the most effective bioinsecticide currently available against mosquitoes. Bti is currently being applied in Third World tropical countries to control mosquito-transmitted endemic diseases (Davidson

et al. 1983).

TABLE 1

Luthy and Eberhold (1981b)

Varieties of *Bacillus thuringiensis*

Serotype	Variety	Serotype	Variety
1	*thuringiensis*	*7*	*aizawai*
2	*finitimus*	*8*	*morrisoni*
3a	*alesti*	*9*	*tolworthi*
3a-3b	*kurstaki*	*10*	*darmstadtiensis*
3a-4b	*sotto*	*11a-11b*	*toumanoffi*
4a-4b	*dendrolimus*	*11a-11c*	*kyushuensis*
4a-4c	*kenyae*	*12*	*tomposoni*
5a-5b	*galleriae*	*13*	*pakistini*
5a-5c	*canadensis*	*14*	*israelensis*
6	*entomocidus*	*15*	*dakota*
6	*subtoxicus*	*16*	*indiana*

There have been two reports of histopathological damage of insect gut epithelia due to Bti toxin. de Barajac (1978), using Bti crystal toxin, examined insect gut epithelia after Bti toxin exposure and found swelling followed by general tissue disruption. Tsiu-Yan (1986) saw condensed mitochondria, loss of ribosomes from endoplasmic reticula, and swelling with vacuoles in the cytoplasm.

Other investigators observed changes in insects, after Bti toxin administration, that indicated muscle and nerve destruction. Luthy (1981a) observed, in *Aedes aegypti* larvae, a slowing of movements and prolonged resting periods, with the larvae finally being unable to control their movements right before death. Singh and Gill (1985) saw muscle sluggishness and blockage of neural synaptic transmission with administration of solubilized Bti crystal protein directly into the gut of the cockroach *Periplanta americana*. In a subsequent study, Singh et al. (1986) used the alkali soluble crystal toxin on the housefly *Musca domestica* and showed myofibril separation from basement membrane, increased vacuole size, and mitochondrial alteration. Chilcott et al. (1984) also saw

inhibition of nerve conduction in the 6th abdominal ganglion of the American cockroach *Periplaneta americana*.

Thomas and Ellar (1983a) examined effects of partially purified Bti toxin in a variety of in vitro and in vivo systems. Bti toxin was toxic to cultured *Aedes albopictus* mosquito cells, insect larvae, human, horse, and sheep erythrocytes, mouse fibroblasts, mouse epithelial carcinoma cells, and primary pig lymphocytes. Bti toxin also killed mice by intravenous and subcutaneous administration. The Bti toxin was not toxic to the lepidopteran larvae (*Pieris brassicae*) and cells (*Choristoneuera fumiferana*) and *B. megaterium* protoplasts. This wide range of mammalian toxicity contrasted with the lepidopteran-specific Bt *kurstaki* delta endotoxin (Bulla et al. 1981), which was only toxic to lepidopteran *C. fumiferana* cells and *P. brassicae* larvae (Thomas and Ellar et al. 1983a), and not to the other types of mammalian cells.

After observing the broad range of eukaryotic cells sensitive to the Bti toxin, Thomas and Ellar (1983b) incubated Bti toxin with a variety of membrane preparations and purified lipids and then assayed for biological activity against *A. albopictus* cells. Bti toxin was inactivated upon incubation with *A. Albopictus* membranes but not by incubation with *B. megaterium* membranes (Thomas and Ellar 1983b). *Bacillus* membranes lack phosphatidyl choline, sphingomyelin, cholesterol, and significant amounts of unsaturated fatty acids (Ellar and Posgate 1973). It was then shown that preincubation with phosphatidyl choline, sphingomyelin, and phosphatidyl ethanolamine liposomes inactivated the Bti toxin in the *Aedes* cell assay. Phosphatidyl serine showed some inactivation and phosphatidyl inositol, cardiolipin, cerebroside, and cholesterol had no effect. Furthermore, unsaturated fatty acid substituents (with the proper head group as noted above) were essential for neutralization of biologic activity (Thomas and Ellar 1983b).

To determine the part of the toxin molecule that was interacting with the lipid, Ellar et al. (1985) incubated Bti toxin with lipid and then digested away toxin not embedded in the lipid with trypsin. Electrophoresis followed by peptide mapping revealed that the 12 Kd carboxy terminal fragment was protected from digestion and therefore must have inserted into the lipid. It was further proposed that the 12 Kd fragment either folds back on to itself to form an amphipathic channel or several 12 Kd fragments from several toxin molecules associate to form the channel which in a cell, would form an opening, disrupting the osmotic balance, causing cell lysis (Ellar 1985). Cytolytic proteins which possess such pore forming activity include the *E. coli* hemolysin (Bhakdi et al. 1986), Colicin El (Cramer et al. 1983), terminal human complement C9 (Stanley et al. 1986), and *Staphylococcus aureus* alpha toxin (Fussle et al. 1981).

Upon alkali solubilization of the Bti toxin parasporal crystal, a number of proteins were revealed by SDS polyacrylamide gel electrophoresis (Figure 2 top, lane a, Armstrong et al. 1985). Earlier investigators (Table 3) used the crude solubilized extract to demonstrate mosquitocidal activity on *A. aegypti* larvae (Schnell et al. 1984, Thomas and Ellar 1983a, Tyrell et al. 1981). The Bti toxin preparation used by Thomas and Ellar (1983a) showed a major electrophoretic band at 26 Kd. Sriram et al. (1985) also used an alkali-solubilized preparation in which a 26 Kd protein predominated and showed larvicidal activity with an LC 50 of 100 ng/ml.

Table 4 shows reports of more purified forms of Bti toxin. Yamamoto et al. (1983) and Armstrong et al. (1985) identified a 28 Kd protein to be the active toxin. Armstrong et al. (1985) then further proteolytically cleaved the 28 Kd molecule to 25 Kd (Figure 2, bottom, lane e and f) and still were able to demonstrate cytolytic activity on mosquito cells and erythrocytes with an LC 50 of 75 ng/ml and larvicidal activity of 50 ug/ml giving 90% lethality. Davidson and Yamamoto (1984) then further isolated the 25 Kd

fragment from their 28 Kd protein and found it to be cytolytic against mosquito cells (LC 100 0.4 ug/ml), erythrocytes (LC 100 0.2 ug/ml) and insect larvae (LC 50 12.5 ug/ml). Armstrong et al's. (1985) and Davidson and Yamamoto's (1984) low larvicidal potency of the 25 Kd protein could have been due to the soluble nature of the purified toxin. Mosquito larvae are filter feeders, concentrating particles 0.7 to 10 microns in diameter (Dodd 1981). Schnell et al. (1984) immobilized Bti toxin on 0.8 micron latex beads and observed a 280-fold increase in toxicity as compared to the solubilized crystals. A 7,000-fold increase in toxicity was observed in comparing intact crystals to solubilized crystals (Schnell et al. 1984).

Recently, there have been a number of reports which describe a 65 Kd protein being mosquitocidal and the 28 Kd protein being only cytolytic or hemolytic (Table 4). Lee at al. (1985) and Hurley et al. (1983) have described 65 Kd proteins that were larvicidal at 180 and 40 ng/ml respectively. Wu and Chang (1985) have seen little larvicidal activity for the 65 and 26 Kd proteins by themselves, but when administered together they saw a synergistic effect. Cheung and Hammock (1985) found mixtures of proteins 25-26 Kd cytolytic and 31-35 Kd with low larvicidal activity (LC 50 6.7 ug/ml). Visser et al. (1986) found the 28 Kd protein hemolytic and found a 130 Kd and 230 Kd protein to be larvicidal. Ibarra and Federici (1986) purified a 65 Kd protein from one of the Bti inclusions and found it larvicidal, but it was 65 times less toxic than the intact crystals themselves; it was concluded that the 65 Kd protein was not larvicidal by itself. Insell and Fitz-James (1985) have determined by separating the various parasporal inclusions, that a mixture of proteins 26.5 and 27 Kd were hemolytic (no concentration given) and larvicidal at 40 ng/ml.

Toxic activity of the Bti delta endotoxin has been shown to be associated with a 75 megadalton plasmid (Gonzalez and Carlton 1984).

Ward et al. (1984) have isolated the 26 Kd protein by cloning fragments of the 75 Md plasmid and have shown the expressed protein to be both mosquitocidal to *A. aegypti* larvae and cytolytic to *A. albopictus* cells. Waalwijk et al. (1985) have also cloned the 28 Kd protein (sequence given in Appendix B) mentioned in Visser et al. (1986) and again found it hemolytic but not larvicidal.

Thus, the literature is not clear as to which protein components alone or together are responsible for the cytolytic or mosquitocidal activity of the Bti toxin. Some of the factors that could contribute to these discrepancies may be related to the method of purification, as some investigators used a gradient to separate spores and crystals, and some used proteolytic enzymes after alkali solubilization. The pH of the protein purification (gel filtration; Table 4) could also have been a contributing factor, as it is known that the 25 Kd protein is stable in the pH range 8 to 9.5 (M. L. Pollock unpublished data), but nothing is known of the pH stability of the 65 or 130 Kd proteins. In addition, the action of proteases in the Bti crystal and in the larval gut must also be taken into account (Chilcott et al. 1983, Garcia-Patrone et al. 1986). The different origin of the strains (Table 4) may also be a contributing factor. Ultimately, all of the strains are traceable back to that of Goldberg and Margalit (1977), but it is not known if the strains studied in various laboratories had a different toxin composition due to plasmid exchange (Carlton and Gonzalez 1985).

The World Health Organization's official bioassay for Bti toxin is on dipteran *A. aegypti* larvae (Richikesh and Quelennec 1983). Bioassays for Bti toxin, reported in the literature, have involved killing of dipteran mosquito larvae such as *A. aegypti* (Tyrell et al. 1979, 1981) lysis of insect cells such as *A. albopictus*, (Thomas and Ellar 1983a, Armstrong et al. 1985) and hemolysis of erythrocytes (Armstrong et al. 1985, Thomas and Ellar 1983a, Sandler et al. 1985, Cheung and Hammock 1985, Visser et al. 1986,

Pfannenstiel et al. 1984, Insell and Fitz-James 1985, Chilcott et al. 1984, Davidson and Yamamoto 1984).

Hemolysis of erythrocytes has been used for over 50 years to study the mode of action of various cytolytic agents (Ponder 1934, 1948). The erythrocyte is a convenient model to study cytolytic phenomena because uniform preparations of cells can be obtained easily in large quantities, the erythrocyte membrane and proteins are well characterized (Bennett 1984), and hemoglobin release upon hemolysis provides a simple method for monitoring cell rupture and leakage of the internal contents. This eliminates more elaborate methods of monitoring cell leakage such as trypan blue exclusion (Thelestam and Mollby 1983), radiochromium (Zawycliwski and Duncan 1978), ^{3}H-2-aminoisobutyric acid (Christensen and Liang 1966), ^{3}H uridine (Thelestam and Mollby 1979), and uptake and release of cellular enzymes such as lactic dehydrogenase (Durkin and Shier 1981).

Organisms such as *Staphylococcus aureus, Streptococcus pyogenes* and *Clostridium perfringens*, as part of their infective process, secrete a number of proteinaceous toxins, some of which are hemolytic (Alouf 1980, Freer and Arbuthnott 1983, McDonel 1980). The alpha toxin of *C. perfringens* is hemolytic and has been shown to be a phospholipase C (Mollby 1978). *S. aureus* alpha toxin is hemolytic and has been shown to form hexameric rings on the erythrocyte membrane surface; these rings are believed to be channels which allow leakage of cellular contents and subsequent cell lysis (Fussle et al. 1981). *Streptolysin* O (from *Streptococcus pyogenes*) is hemolytic, is inhibited by cholesterol, requires the cleavage of disulfide bonds for activity, and shows pit formation on electron micrographs, suggesting channel formation (Bernheimer 1974, Bhakdi et al. 1984). Other hemolytic toxins have been shown to bind the erythrocyte membrane and elicit specific pharmacological responses such as the marine coelenterate palytoxin which binds the Na^{+}/K^{+} ATPase causing disruption of the pump and subsequent cell lysis (Ozaki et al.

1985).

J. Armstrong (unpublished data) studied the effect of various ions on Bti toxin hemolysis. Cells did not lyse in the presence of toxin in a 20 mM Tris 150 mM sucrose pH 7.5 buffer. However, upon the addition of either 8 mM Mg^{++}, 10 mM Na^{++}, 20 mM K^{+}, or 40 mM NH_4^{+}, complete lysis occurred. Addition of Ca^{++}, Mn^{++}, Cu^{++}, and Co^{++} had no effect. In inhibition studies, it was found that 2 uM La^{+3}, 150 uM Cu^{++}, 200 uM Zn^{++}, 5 mM Co^{++}, 10 mM Mn^{++} and 20 mM Ca^{++} inhibited lysis 80%. This rank order of potency in blocking hemolysis ($La^{+++} > Zn^{++} > Mn^{++} > Ca^{++}$) is similar to that seen for metal blockage of the calcium channel (Hagiwara and Takahashi 1967a); however, it is not known whether erythrocytes have such a channel (F. Vincenzi, personal communication).

Pore-forming *Staph* alpha toxin hemolysis has been reported to be blocked by 30 mM Ca^{++} (Harshman and Sugg 1985). In a voltage-dependent inhibition study of *Staph* alpha toxin with planar lipid membranes, Menestrina (1986) found metal inhibition in the order of potency: $Zn^{++} > Tb^{+3} > Ca^{++} > Mg^{++} > Ba^{++}$, suggesting that carboxyl groups of the 34 Kd *Staph* alpha toxin molecule could be situated on the outer surface of the channel to which the metal ions can bind.

THESIS PLAN AND SIGNIFICANCE

The literature to date on the hemolytic activity of the Bti toxin has been concerned only with using hemolysis as an assay for gross cytolytic activity. There is little information on the specific interaction of the toxin with the erythrocyte. This thesis examined the interaction of the Bti toxin and the erythrocyte membrane by establishing the role of toxin concentration, cell concentration, pH, potassium release, and temperature on the hemolytic process. In addition, the effects of various substances and cell treatments on Bti toxin hemolysis were examined, in an effort to reveal the specific interaction of the toxin and the cell membrane. There have been no membrane binding studies reported for Bti toxin, so a fluorescence binding study was done. Bti toxin was assayed for phospholipase C activity, as other hemolytic bacterial toxins have shown this activity.

In purifying the Bti toxin, Armstrong et al. (1985) used trypsin and proteinase K for proteolytic treatment of the alkali-solubilized crystal protein. They found that the remaining 25 Kd protein was resistant to these proteolytic enzymes although amino acid analysis showed the presence of cleavage sites for both enzymes. To understand properties of the thermodynamic stability of the Bti toxin that confers protease resistance, denaturation and renaturation studies were carried out with heat and urea. The effect of the denatured and renatured toxin was studied on insect cells and erythrocytes. Finally, the tryptophan content of the Bti toxin molecule was determined, as this was not done in the amino acid analysis of Armstrong et al. (1985).

Bti toxin possesses a wide range of cytotoxicity to mammalian cells, in addition to its narrow range of specificity to insect cells. It is possible that both activities rely on a common mode of action. The erythrocyte has been chosen to study the mechanism of action of

the Bti toxin because it is a more easily manipulatable system. The results obtained from this system must then be examined with insect cells, in order to determine if indeed the mechanism of action of the Bti toxin is the same in both cell systems.

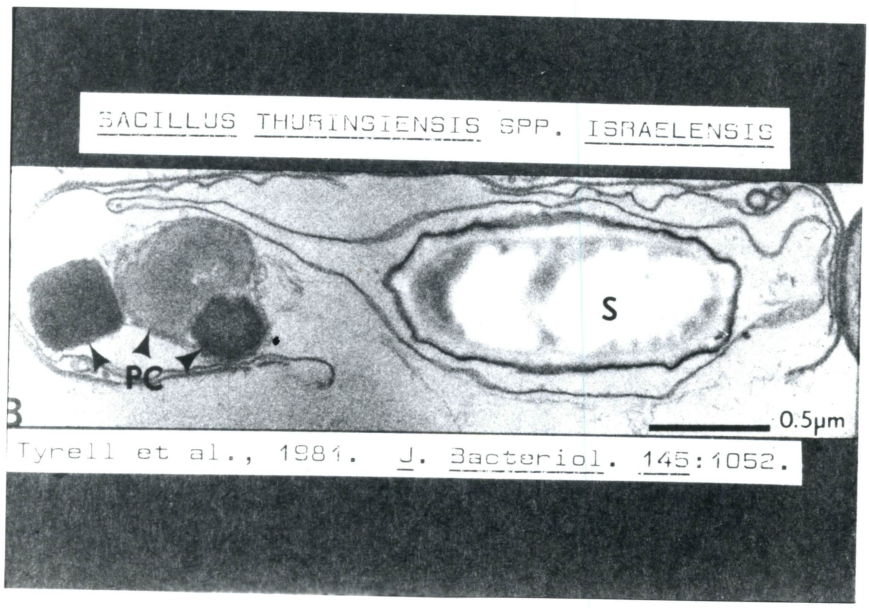

Figure 1. Toxin containing parasporal crystals of Bti.

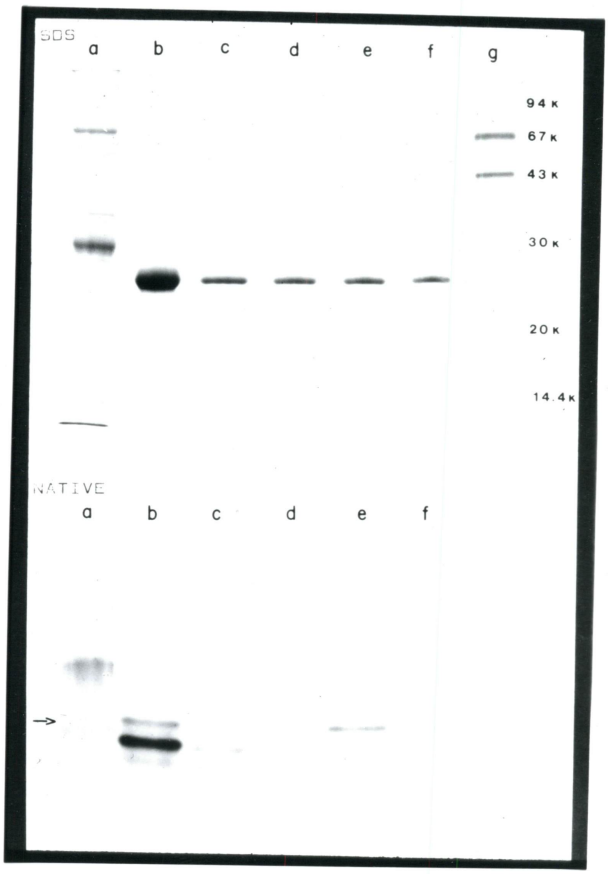

Figure 2. Alkali solubilized proteins of Bti.

TABLE 2

Important Insects Susceptible to *Bacillus thuringiensis*, Together With Host Crops

Insect Species	Crops
Trichoplusia ni (Cabbage looper)	vegetables, soybean, tobacco
Plutella maculipennis (Diamond black moth)	vegetables
Pieris brassicae (Large white butterfly)	vegetables
Pieris rapae (Imported cabbage looper)	vegetables
Heliothis zea (Bollworm)	soybean, tobacco
Heliothis virescens (Tobacco budworm)	soybean, tobacco
Ostrinia nubilalis (European corn borer)	corn
Manduca sexta (Tobacco hornworm)	tobacco
Lymantria dispar (Gypsy moth)	forest
Choristoneuera fumiferana (Spruce budworm)	forest
Malacosoma disstria (tent caterpillar)	forest
Dendrolimus sibiricus (Siberian silkworm)	forest
Plodia interpunctella (Indian meal moth)	stored grain
Ephestia cautella (Almond moth)	stored grain
Anagasta kuehniella (Mediterranean flour moth)	stored flour
Aedes aegypti	vector of yellow fever
Anopheles stephensi	vector of malaria

Luthy and Ebersold (1981b)

TABLE 3

Solubilized Bti Crystal Protein

Ref.	Cytotoxicity LC_{50} (ug/ml)	Cell type	Protein size(Kd)	Larvicidal LC_{50} (ug/ml)	insect species	Strain	Purification spore crystal protein	pH gel filtration	Comments
Srivan et al. ('85)			26	0.19.	tritraen nirhynehus	HdeB	+		Immobilized trypsin
Schnell et al. ('84)				0.19	A. aegypti	S. Sirgen (West. Ill. Univ.)	+		Immobilized
Ellar et al. ('83)	1.5	RBC		0.05	A. aegypti	HdeB	+		
Tyrell et al. ('81)				0.19	A. aegypti	Abbott	+		

TABLE 4

Purified Bti Protein

Ref.	Cytotoxicity Protein size(Kd)	Cytotoxicity LC_{50} (ug/ml)	Cytotoxicity Cell type	Larvicidal Protein size(Kd)	Larvicidal LC_{50} (ug/ml)	Larvicidal insect species	Purification Strain	Purification spore-crystal separation	Purification pH gel filtration	Comments
Lee et al. ('85)				65	0.18	A. aegyptus	-	+		separate inclusions
Hurley et al.('85)				65	0.04	A. aegyptus	Abbott	+	8	
Yamamoto et al.('83)				28	0.03	A. aegyptus	Goldberg		8	proteinase K
Davidson and Yamamoto ('84)	25	0.4* 0.2*	C. quin# ery+	25	12.5	C. quin.	Goldberg		8	
Armstrong et al. ('85)	25	0.075 0.075	ery A. albopictus	25	50	C. pipiens	Abbott		8.5	trypsin proteinase K
Wu and Chang ('85)				65 26 65 26	1.6 6.4 0.1++ 0.4++	A. aegyptus	Bacillus Genetic Stock Ctr. Columbus, OH	+	100 mM NaOH	immobilized
Cheung and Hammock ('85)	25 25/26	0.85 1.6	ery	31/34/35	6.7	A. aegyptus	Carlton Univ. GA	+	8	
Visser et al. ('86)	28		ery	130 230	0.04	A. stephensi	HdeB@	+	9.5	immobilized
Ibana et al. ('86)				65	0.043	A. aegypti	HdeB	+		separate inclusions
Insell and FitzJames ('85)	26.5/27		ery	26/27.5	0.04	A. aegypti	O. Lysenko Prague, Czechoslovakia		-	separate inclusions
Sekar ('86)				130	0.025*	A. aegypti	H. Dulmage USDA, Brownsville, TX	+		crystal inclusions

++ = LC_{50} for synergistic mixture
# Culex quinquefasciatus
* LC 100
@HdeB = H. de Barjac, Institute Pasteur
+ erythrocyte

MATERIALS AND METHODS

Bti Toxin

Bti toxin used was the 25,000 Kd ("25a") protein isolated and purified according to Armstrong et al. (1985). The source of Bti was an industrially produced culture powder supplied by Abbott Laboratories (North Chicago, IL). The powder was treated with alkali to solubilize the crystals; the proteins released were then proteolytically degraded with trypsin and proteinase K. This was followed by ammonium sulfate precipitation, gel filtration and ion exchange chromatography. The purified protein showed only a single band using silver stain with native and denaturing polyacrylamide gel electrophoresis (Figure 2).

Aedes albopictus Mosquito Cell Bioassay

The *Aedes albopictus* mosquito cell bioassay was performed in duplicate as described in Armstrong et al. (1985). *Aedes albopictus* cells (from Yale Arbovirus Unit, Yale Medical School, New Haven, CT) were maintained at 28°C in 25 ml plastic flasks containing 5 ml of medium whose composition is given in Appendix A. When cells reached 60-80% confluence, the medium was removed and 2.5 ml of fresh medium previously warmed to 28°C was added and the cells were detached by hitting the flask against the table edge. Two drops of the cell suspension were distributed to wells of a 96 well flat-bottom microtiter plate and incubated for 1 hour at 28°C to permit attachment. The medium was then removed and each well washed with 100 ul of PBS (0.09% Na_2HPO_4, 0.02% KH_2PO_4, 0.8% NaCl, 0.02% KCl, pH 7.0). The assay was begun by adding Bti toxin (8 to 40 nM) in 100 ul of PBS containing 0.025% trypan blue. The assay was terminated after one hour at 28°C by removing the toxin followed by a wash with 100 ul of PBS. Cytolysis was examined under a Nikon inverted microscope and subjectively graded on a scale of 0 (no lysis) to 5

(100% lysis). The degree of toxin inactivation was estimated by determining the excess of inactivated toxin that had to be added to the assay to observe the same lysis effect as the control.

Preparation of Blood for Hemolysis Bioassay

Fresh human blood (obtained from Kings Road Medical Laboratory, Corvallis, OR) was stored on ice at 4°C and used within one week. The blood was washed 3 times by dilution in 100 volumes of 20 mM Tris 150 mM NaCl 3 mM EDTA buffer with centrifugation for 5 minutes at 1,500 x g. The pellet was resuspended in 20 mM Tris 150 mM NaCl pH 7.4 (T/N) buffer to give a cell concentration of 2.5×10^8 cells/ml (equivalent to a 2% hematocrit). The washed erythrocyte suspension was stored on ice and used for one day only.

Fixed-Time Hemolytic Bioassay

For the fixed-time hemolytic bioassay, duplicate samples of toxin (4 to 8 nM) were incubated with erythrocytes at 2.5×10^7 cells/ml (1 X cells) in T/N buffer at 37°C for 60 minutes with gentle shaking every 15 minutes. The reaction was carried out in a 1.0 ml volume. At the end of 60 minutes, the suspension was centrifuged in an Eppendorf 5914 microfuge at 12,000 x g for 20 seconds. The extent of hemolysis was determined by hemoglobin absorbance in the supernatant, at 540 nm, on an LKB 4050 spectrophotometer (Harshman et al. 1985). Total (100%) hemolysis was induced in the assay by addition of excess toxin (32-40 nM). All assays included a blank, consisting of all the ingredients minus the toxin.

Kinetic Hemolytic Bioassay

For the kinetic hemolytic bioassay, toxin and cells were incubated under the same conditions as above, except that hemolysis was measured at 0, 10, 20, 30, 40, 50, and 60 minutes. Either (1), a

large quantity of cells (e.g. 16 ml) and toxin were incubated and aliquots were withdrawn at the different time intervals, centrifuged and the supernatant removed, or (2), multiple tubes were incubated, each containing 1.0 ml of toxin plus cells, and removed for centrifugation at the different time intervals. Controls were also run as described.

The fixed-time and kinetic hemolytic bioassay were used to study the toxin-erythrocyte interaction under various conditions. Variations in these assays were made according to the particular experiment and these will be noted.

Buffers for pH Studies

The buffer used for the pH studies was the Britton-Robinson universal pH buffer (150 mM NaCl, 6 mM H_3BO_3, 6 mM CH_3COOH, and 6 mM H_3PO_4) adjusted with 1 N NaOH to pH 5, 6, 7, 8, 9, and 10 (Habermann, 1981).

Potassium Release Measurement

Potassium in the hemolysate was measured on a Perkin Elmer Model 403 atomic absorption spectrophotometer set for the emission mode at a wavelength of 766.5 nm. The percent potassium release was expressed relative to the amount determined at 60 minutes.

Inhibition Studies

Ascites fluid containing a monoclonal antibody (MAB) to the Bti toxin produced by Armstrong et al. (1985) was purified by Rebecca Russell (G.S. Beaudreau laboratory) using Bio-Rad CM-Affi-Gel Blue and ammonium sulfate precipitation (Bio-Rad 1981, 1984) and assayed with an ELISA by Rebecca Russell. Tetraethylammonium chloride (TEA), verapamil, tetrodotoxin, 4,4'Diisothiocyano-2,2'-stilbene

disulfonic acid (DIDS), furosemide, and ouabain were obtained from Sigma Chemical Co. (St. Louis, MO).

ATP-Depleted Erythrocytes

Erythrocytes were depleted of ATP by incubating washed 10 X cells in T/N buffer for 20 hours at 37°C (Feo and Mohandas 1977). The cells were then washed twice and resuspended with T/N buffer at a 10 X cell concentration.

Neuraminidase Treatment

3 ml of washed 10 X cells were centrifuged and resuspended in 3 ml of 0.9% NaCl containing 10 mM $CaCl_2$ and 0.8 units (N-acetyl neuraminic acid-lactose)/ml of neuraminidase (Sigma N-2876 EC 3.2.1.1.8) and incubated at 37°C for 60 minutes (Prigent 1983). The cells were then washed 3 times with 1 ml and resuspended in 3 ml of T/N buffer. Control cells were treated as above but without neuraminidase.

Chicken Erythrocytes

Chicken erythrocytes were obtained from the O.S.U. Poultry Science Department and were washed and diluted as previously described under Preparation of Blood.

FITC labelling of Bti Toxin

Bti toxin was conjugated with fluorescein isothiocyanate (FITC) on Celite (Sigma F-1628) at a FITC/lysine ratio of 3.6/1. Toxin (8.8 uM) was incubated with 2.5 mg of FITC-Celite in 50 mM Sodium Carbonate buffer pH 8.5 for 3 minutes at 25°C (Rinderknecht 1962). The mixture was centrifuged and the supernatant was chromatographed over a 1.5 X 5.5 cm Sephadex G-25M gel filtration column using 20 mM

sodium phosphate buffer pH 6.5 as an eluent. 0.5 ml toxin containing fractions, located by bioassay, were pooled. The ratio of bound FITC to toxin molecules was calculated according to Goldman (1968).

Urea and Heat Denaturation Studies

Urea (Sigma U-1250) was prepared 10 M in water, and de-ionized to remove cyanates (Hagel et al. 1971) by passage over a 2.5 X 3 cm AG501 x 8 (Bio-Rad) analytical mixed-bed resin with water as the eluent. Typically 50 ml was passed through the column in which the middle third of the effluent was used. The de-ionized urea was kept at 4°C and discarded after 1 week. Refractive index measurements (Bausch & Lomb Refractometer ABBE-3L) of different 10 M urea batches were consistently close to 1.40.

Intrinsic fluorescence measurements were performed on a Perkin Elmer 6510S fluorescence spectrophotometer with toxin concentration always at 400 nM and the excitation wavelength was 280 nm. Protein measurements were by the method of Lowry as modified by Peterson (1983). Coomassie blue dye binding measurements were performed with the Bio-Rad (Richmond, C.A.) protein assay reagent which is based on the method of Bradford (1976). Polyacrylamide gel electrophoresis was performed according to Maizel (1971) with a 3% stacking gel and a 12% running gel for denaturing gels and a 7% for native gels.

Biotinylation of Bti Toxin

Bti toxin was biotinylated at a 10/1 D-biotin-N-hydroxy-succinimide ester/toxin ratio according to Bayer et al. (1979).

Phospholipase C Determination

Bti toxin was assayed for phospholipase C (EC 3.1.4.3) activity by

the egg yolk turbidimetric method of Ottolenghi (1969). The yolk of one egg was washed with 6 portions of 100 ml of 0.9% NaCl. The yolk was then emulsified in 200 ml of 0.9% NaCl and left to stand for 1 hour to allow for the heavier particles to sediment down. The emulsion was then filtered through a 5 cm Seitz filter and NaCl was added to 2% concentration and stored at 4°C. Phospholipase C activity was demonstrated by the increase in turbidity of the emulsion due to lipid breakdown. Phospholipase C from *C. perfringens* (Sigma P7633) was used as the control.

Spectroscopic Determination of Tryptophan and Tyrosine

The tryptophan and tyrosine content of Bti toxin was determined by the method of Edelhoch (1967). Bti toxin (12.7 uM) was dissolved in 20 mM phosphate 6 M guanidine HCl pH 6.5 and incubated at 25°C for 3 hours. Absorption measurements were made at 288 and 280 nm with a Varian Cary 219 recording spectrophotometer. Solution of two simultaneous equations based on the molar absorption coefficients of the model compounds N-acetyl-l-trytophanamide and glycine-l-tyrosyl-glycine yielded the number of tryptophans and tyrosines in the toxin molecule. Chicken egg white lysozyme (18 uM) (Sigma L6876) and RNAse A (92 uM) (Sigma R4875) were also analyzed as controls as described above.

RESULTS

Hemolytic Bioassay

As a convenient and rapid method for screening the effect of different compounds and conditions on the Bti toxin-erythrocyte interaction, the extent of hemolysis at the end of 60 minutes was monitored. The results of the fixed-time hemolytic bioassays with toxin concentrations of 0.44, 1, 2, 4, 8, 12, and 16 nM are given in Figure 3. 100% hemolysis in the assay typically gave an A_{540} of 0.6 and was equivalent to 100% hemolysis produced by hypotonic lysis (20 mM Tris no NaCl). The cells were stable during the fixed-time hemolytic bioassay, as a typical A_{540} of the blank was 0.02 or only 3% of the 100% hemolysis absorbance. To study the effect of various conditions and compounds on Bti toxin hemolysis, 4 to 8 nM of toxin was chosen because this concentration consistently produced between 80-100% hemolysis in the fixed-time hemolytic bioassay.

To study differences in the kinetics of the Bti toxin-erythrocyte interaction produced by various substances and treatments, the extent of hemolysis was determined every 10 minutes over a time period of 60 minutes. The results of a typical kinetic hemolytic bioassay are presented in Figure 4. The 4 nM toxin (top curve) assay plateaued to 100% hemolysis at 50 minutes. There was a lag of 10 minutes before any hemolysis was detected.

Effect of Bti Toxin Concentration on Hemolysis

In order to determine the effect of Bti toxin concentration on on erythrocyte lysis, fixed-time hemolytic bioassays were carried out with toxin concentrations varying from 0.44 to 16 nM as shown in Figure 3 and described above under Hemolytic Bioassay. Lysis started at 1 nM (26%) and proceeded in a linear fashion to 4 nM (96%) at which point it plateaued out. At 4 nM toxin concentration,

the toxin-to-cell ratio was 96,320. In order to determine the relationship between the time to achieve partial hemolysis and toxin concentration, kinetic hemolytic bioassays were carried out with toxin at 1, 2, 4, 8, and 16 nM concentrations. Figure 5 shows the time to achieve 25% hemolysis at each concentration. The reaction was very slow at 1 nM taking the entire 60 minutes to achieve 25% hemolysis. At a toxin concentration of 2 nM, the reaction proceeded faster with 25% hemolysis achieved at 14 minutes. At 4 nM toxin concentration, the time to achieve 25% hemolysis plateaued, which agreed with Figure 3. In another experiment, a kinetic hemolytic bioassay was carried out with toxin at 4 and 0.44 nM concentrations (Figure 4). Toxin concentration of 0.44 nM produced only 4% lysis in 60 minutes.

Effect of Cell Concentration on Bti Toxin Hemolysis

If the Bti toxin-erythrocyte membrane interaction was similar to a pharmacologic ligand-receptor interaction, with a finite number of high-affinity binding sites, then increasing the number of receptors (erythrocytes) should decrease the extent of hemolysis, if the number of receptors are in excess of the number toxin molecules. (Burt 1978). Therefore, the effect of cell concentration on Bti toxin hemolysis was examined. A kinetic hemolytic bioassay was carried out in which the number of cells was varied over an 18.4-fold concentration. Assays were run at 0.5, 5 and 9.2 X cell concentrations, corresponding to 1.25×10^7, 1.25×10^8, and 2.3×10^8 cells per ml respectively. Each assay was run with a standard cell concentration (2.5×10^7 cells per ml) as a control. Toxin concentration was 4 nM for all assays. For the 5 X and 9.2 X assays, the supernatants were diluted with T/N 5 and 9.2 times respectively before absorbance readings were taken. Varying the cell concentration over the 18.4-fold range did not effect the rate or per cent of hemolysis, as the 0.5, 1, 5, and 9.2 X curves were similar in appearance (Figure 6).

In order to standardize conditions for the four different cell concentrations, fixed-time hemolytic bioassays were carried out in which 4 nM toxin was incubated with 0.5, 1, 5, and 9.2 X cells and over this cell concentration range, hemolysis was linear, as every new cell added was lysed (Figure 7). These results agreed with those of Figure 6. At a saturating concentration of toxin (4 nM) (Figure 3), for 2.5 X 10^7 cells, it was possible to lyse 2.3 X 10^8 cells. The toxin could have been recycling, or the cells were driving the reaction in some manner.

Effect of Changing Bti Toxin to Cell Ratio by Lowering Toxin Concentration

The data in Figures 6 and 7 showed that from 0.5 to 9.2 X cell concentration at 4 nM toxin concentration, the extent of hemolysis was independent of the number of cells present. In order to determine if it was the toxin concentration that was critical for a toxin-erythrocyte interaction, a toxin-to-cell ratio of 10,595 (similar to the ratio of 10,470 that gave a full hemolysis with the 9.2 X cell concentration in Figures 6 and 7) was established by using a 1 X cell concentration and lowering the toxin concentration to 0.44 nM. A kinetic hemolytic bioassay was carried out at a toxin concentration of 0.44 nM and 4 nM. Figure 4 shows that the 0.44 nM assay produced only 4% lysis in 60 minutes which was very much different from the 100 percent hemolysis produced in the 9.2 X cell concentration assay in Figure 6. It was the concentration of toxin in solution that was critical for the hemolytic reaction to occur in 60 minutes.

Effect of Bti Toxin Incubation with Lysate on Hemolysis

In order to determine if the Bti toxin was recycled after the erythrocyte interaction, supernatant (containing toxin) from an assay was added to fresh cells to see if lysis would occur. There

was substantial (50%) loss of activity of the "used" toxin (data not shown). To ascertain if something in the lysate was causing the activity loss, a 1 X and 9.2 X erythrocyte lysate were prepared by hypotonic lysis in 20 mM Tris pH 7.4 buffer. The membranes were removed by centrifugation (1500 g X 5 minutes) and NaCl added to the supernatant to a final concentration of 150 mM. Toxin was then incubated with the 1 X and 9.2 X lysate for 10 and 60 minutes at 37°C. At the conclusion of these assays, a fixed-time hemolytic bioassays were run. Preincubation with toxin and erythrocyte lysate caused an apparent inactivation of the toxin, more so with the 9.2 X lysate (Table 5). With the 60 minute preincubation, only 4% hemolytic activity remained with the 9.2 X lysate as compared to 31% hemolytic activity for the 1 X lysate (Table 5). A similar result with *Staph* alpha toxin incubation with rabbit erythrocyte lysate was also observed by Cooper et. al. (1964b). The rapid loss of hemolytic activity vs. time and lysate concentration (Table 5), suggested enzymatic degradation of the toxin. Another possibility was that the toxin was being inactivated by binding to membrane fragments, which due to their small size, were not removed by centrifugation.

TABLE 5

Effect of Erythrocyte Lysate on Bti Toxin Hemolysis

Incubation Lysate	1 X	9.2 X
Time (minutes)	% Hemolysis	
10	86	31
60	66	4

Effect of pH on Bti Toxin Hemolysis

Bti toxin is active in the alkaline dipteran gut, and it was desired to know if the hemolytic activity curve at different pH's would reflect this. In order to study the effect of pH on the toxin-erythrocyte interaction, Bti toxin (8 nM) was incubated at pH 5, 6, 8, 9, and 10 as described in the kinetic hemolytic bioassay above. Each pH was a separate experiment and was run with a pH 7 control (Figures 8 to 12). There was a definite reduction in biological activity at pH 5 and pH 10 (Figure 8, 12). The biologic activity from pH 6 to 9 did not appear to vary much as the initial slopes of hemolysis were similar to the pH 7 control (Figures 9-11). Erythrocytes with no toxin showed no lysis from pH 5 to 10 under the conditions of the assay. However, the erythrocytes did start to lyse at pH 4.5 and pH 10.5.

In order to standardize conditions for the individual pH experiments of Figures 8 to 12, a fixed-time hemolytic bioassay was performed at 8 nM toxin concentration at pH 5, 6, 7, 8, 9, 9.5, and 10. The data point of pH 9.5 was included to determine with greater accuracy the pH at which the loss of hemolysis was occurring. The results are shown in Figure 13 in which the percent hemolysis is relative to pH 7. Figure 13 shows a sharp loss of hemolysis occurring between pH 9.5 and 10.

Effect of pH on Erythrocytes

It was possible that the loss of hemolytic activity seen at pH 5 and 10 was due to the cells being irreversibly altered such that they lost their ability to respond to the action of the Bti toxin. To determine this, cells were incubated at pH 10 and 5 and then the pH was changed to 9 and 6 respectively and assayed for their responsiveness to toxin. 1 X cells were incubated for 60 minutes at pH 10 at 37°C then toxin at 8 nM was added and a fixed-time

hemolytic bioassay carried out. Another aliquot of these pH 10-treated cells was centrifuged at 1,500 g x 5 minutes, the supernatant was removed, and the cells were resuspended with pH 9 buffer. Toxin at 8 nM was added, and at 0 time and after 30 minutes, fixed-time hemolytic bioassays were then carried out. The control was toxin (8 nM) plus cells incubated at pH 9 at 37°C for 60 and 90 minutes. A similar experiment to the pH 10 to 9 described above was carried out with cells going from pH 5 to 6 (control was toxin plus cells at pH 6). The results are shown in Table 6.

TABLE 6

Effect of pH 10 and pH 5 on Susceptibility of Erythrocytes to Bti Toxin Lysis

	Bti toxin plus erythrocytes treated at pH 10 then pH 9
Cell treated at	% Hemolysis
pH 10 x 60 minutes then add toxin	9
pH 10 x 60 minutes change to pH 9 then add toxin	95
pH 10 x 60 minutes change to pH 9 for 30 minutes then add toxin	98

	Bti toxin plus erythrocytes treated at pH 5 then pH 6
	% Hemolysis
pH 5 x 60 minutes then add toxin	0
pH 5 x 60 minutes change to pH 6 then add toxin	96
pH 5 x 60 minutes change to pH 6 for 30 minutes then add toxin	96

Treating the cells at the extreme pH's of 10 and 5 in which they were not responsive to toxin, did not irreversibly alter their ability to respond at pH 9 and 6. The control toxin at pH 6 and pH 9 maintained full activity during the course of this experiment.

Effect of pH on Bti Toxin

In order to study the reversibility of the loss of hemolytic activity seen at pH 10, Bti toxin (4 nM) was incubated at 37°C for 60 minutes at pH 10 and a fixed-time hemolytic assay was done at 4 nM. The pH of the toxin then was adjusted to 9 with 6 M HCl and fixed-time hemolytic assays were done at 0, 10 and 60 minutes. The control was Bti toxin at pH 9. Table 7 shows that there was an initial restoration of biological activity, but this was rapidly lost. The Bti toxin was denatured at pH 10, and a fully renatured and biologically active molecule was not seen.

TABLE 7

pH 10 Denaturation and Renaturation of Bti Toxin

	% Hemolysis
Toxin pH 10 X 60 minutes	4
Then Change to pH 9 for:	
0 minutes	73
10 minutes	44
60 minutes	38

Effect of Temperature on Bti Toxin Hemolysis

To determine if the Bti toxin-erythrocyte interaction was temperature dependent below 37°C, a kinetic hemolytic bioassay

(toxin concentration 4 nM) was performed at 37, 20, and 0°C. The extent of Bti toxin hemolysis decreased as the temperature was lowered below 37°C (Figure 14). At 20°C, only 50% hemolysis was achieved in 60 minutes. Also the lag time increased from 5 minutes at 37°C to 30 minutes at 20°C. At 0°C, hemolysis was negligible (2-3%) throughout the 60 minute incubation. To study the effect on the toxin-erythrocyte interaction of raising the temperature above 37°C, another kinetic hemolytic bioassay (toxin concentration 4 nM) was performed at 37° and 47°C. Raising the temperature to 47°C did not change the rate of hemolysis as compared to 37°C as the kinetic hemolytic bioassay curves for the two temperatures were similar in shape (data not shown). The erythrocytes without toxin showed no significant lysis as compared to the control at all of the temperatures studied.

Relationship of Potassium Release to Hemoglobin Release in Bti Toxin Hemolysis at 37°C

Bti toxin hemolysis could proceed by first creating a small membrane lesion, by which small ions could leak out, causing the lesion to increase in size, after which the larger hemoglobin molecule egresses. Therefore the small ion potassium, which is intrinsically present in the erythrocyte, should be able to be detected before hemoglobin. In order to accumulate more data points for kinetic analysis than the usual 7 points of the standard assay of Figure 4, a kinetic hemolytic bioassay (toxin concentration 8 nM) was performed with points taken at 1, 5, 7, 9, 11, 13, 15, 17, 19, 21, 24, 29, 36, 45, 55, and 60 minutes, and the supernatant was assayed for potassium in addition to hemoglobin (Figure 15). From the time period of 5 to 9 minutes, the amount of potassium in the supernatant was more than twice that of hemoglobin. For the 11, 13 and 15 minute points of Figure 15, the ratio of potassium to hemoglobin was 2.0, 1.5, and 1.3 respectively.

Figure 16 shows a first order rate plot of the data from Figure 15. The release of hemoglobin appeared to follow a single phase first order efflux with a half-time of 6.0 minutes. The potassium release appeared to be multiphasic with an initial rapid first order efflux (half-time 3.3 minutes) followed by one or more slower components (Figure 16).

Effect of Calcium on Bti Toxin Hemolysis

J. Armstrong (unpublished data) found that hemolysis was 80% inhibited when trivalent and divalent metals were present during a fixed-time hemolytic bioassay (toxin concentration 8 nM) at the following concentrations:

Metal	Concentration (mM)
La^{+++}	0.002
Cu^{++}	0.15
Zn^{++}	0.2
Co^{++}	5
Mn^{++}	10
Ca^{++}	20

The effect of calcium in the concentration range of 0.1 to 100 mM on the Bti toxin erythrocyte interaction was studied. Figure 17 shows a fixed-time hemolytic bioassay with toxin concentration of 8 nM and $CaCl_2$ concentration of 10, 20, 30, 40, and 50 mM. The results confirmed the data of J. Armstrong (unpublished) showing that calcium can cause a reduction in Bti toxin hemolysis at millimolar concentrations. 100 uM calcium had no effect on hemolysis (data not shown), 1 mM calcium was inhibitory and gave 70% hemolysis, 10 mM gave 21% hemolysis, 40 mM gave 6% hemolysis, and 100 mM (data not shown) calcium gave 2% hemolysis.

Effect of Calcium on Erythrocytes

It was possible that the loss of hemolytic activity seen in Figure 17 was caused by the calcium irreversibly altering the cells such

that they lost their ability to respond to the action of the Bti toxin. To determine this, 1 X cells were incubated with 40 mM of $CaCl_2$ for 30 minutes at 37°C. The supernatant was removed, the cells were resuspended with fresh buffer, and a fixed-time hemolytic bioassay was carried out at a toxin concentration of 8 nM. The control cells were incubated as above, except with no calcium. After preincubation of the cells with 40 mM calcium, and after removal of the calcium, the toxin was still able to lyse 93% of the cells. Therefore, 40 mM calcium did not cause an irreversible change in the cells, such that they could not respond to toxin.

Effect of Calcium on Bti Toxin

The loss of hemolytic activity seen in Figure 17 could also have been caused by the calcium irreversibly inactivating the toxin. To determine this, Bti toxin (10 uM) was incubated with 40 mM of $CaCl_2$ for 30 minutes at 37°C and the toxin was then added to a fixed-time hemolytic bioassay. The toxin concentration in the assay was 8 nM, causing the toxin to be diluted 1245 times such that the effective calcium concentration was 30 uM. The control was toxin treated as above, except with no calcium. After the toxin was preincubated with 40 mM calcium followed by removal by dilution, the toxin was still able to lyse 88% of the cells. Thus, 40 mM calcium did not cause an irreversible inactivation of the toxin.

Effect of Calcium on Bti Toxin Hemolysis Added at Various Times During the Hemolytic Bioassay

In order to determine if calcium could inhibit Bti toxin hemolysis after it had begun, fixed-time hemolytic bioassays were run at a toxin concentration of 8 nM, and during the course of the assays, to separate tubes, 40 mM $CaCl_2$ was added at 5, 10, 15, 20, and 25 minutes. The assays were then continued for an additional 60 minutes. Figure 18 shows that addition of 40 mM calcium to the

hemolytic bioassays at any time from 5 to 25 minutes completely prevented any additional lysis. The amount of hemolysis seen after 40 mM calcium addition was close to the hemolysis seen for the standard kinetic hemolytic bioassay at 8 nM toxin concentration to which no calcium was added (plotted as "no calcium" in Figure 18). This indicated that no further hemolysis was taking place after calcium addition and that the time of the calcium addition during the assay was not critical.

Estimation of the Binding to Bti Toxin to Erythrocytes

The strength of any Bti toxin-erythrocyte binding that may have been taking place during the lag period of the kinetic bioassay (Figure 18) was estimated. In addition, the effect of calcium on inhibiting this binding after it had already taken place was studied. A fixed-time hemolytic bioassay was started at a toxin concentration of 8 nM and after 5 minutes, the cells were centrifuged, the supernatant removed, and the hemolysis determined. The pellet was resuspended with fresh T/N buffer and the assay continued for 60 minutes. The above experiment was repeated except the resuspending buffer contained 40 mM $CaCl_2$. The control was an assay in which the cells were not resuspended. Minimal lysis (0-2%) was seen in the supernatant during the first 5 minutes of the fixed-time hemolytic bioassay (Table 8). However, when fresh buffer was added and the bioassay continued for 60 minutes, still, very little lysis was seen (13%). Resuspension with 40 mM calcium showed slightly less hemolysis (10%).

In order to estimate the strength of toxin-erythrocyte binding after the lag period of the kinetic bioassay in Figure 18, a similar experiment was conducted as above, except the time of initial toxin plus erythrocyte incubation was 10 minutes and resuspension was without calcium. When the hemolytic bioassay was disrupted at 10 minutes, 22% hemolysis had already taken place; when the assay was

continued with fresh buffer, only another 22% of the potential 78% of the hemolysis was observed (Table 8). If there was any binding of toxin to the cells, it was very weak as removing the supernatant appeared to have washed the toxin away.

TABLE 8

Effect on Hemolysis of Removing Bti Toxin During Hemolytic Bioassay

Toxin + Cells Incubation minutes	% Hemolysis Supernatant	% Hemolysis Resuspend & Incubate
5	0	13
10	22	22
		Resuspend & Incubate with 40 mM Calcium
5	2	10

Effect of DTT, EDTA, EGTA, ATP, Magnesium, BSA, Sucrose, and KCl on Bti Toxin Hemolysis

A number of substances and treatments were studied to gain insight into the nature of the interaction with the Bti toxin and the erythrocyte (Table 9): DTT to determine if there was need for intact disulfide bonds; EDTA and EGTA to determine if there was need for divalent ions; sucrose and Bovine Serum Albumin (BSA) to determine if hemolysis could be blocked osmotically; ATP to determine if there was need for positively charged sites on the toxin or the erythrocyte membrane surface; $MgCl_2$ to compare with the other divalent metals that have been determined to be inhibitors of hemolysis (mentioned in Effect of Calcium on Bti Toxin Hemolysis above); and KCl to determine the effect of substitution of another monovalent ion for sodium on the hemolytic process.

Fixed-time hemolytic bioassays were carried out with the above substances, some with a 15 minute preincubation with the cells, as indicated in Table 9. Table 9 also gives the concentrations of the substances in the assay as well as the concentration of toxin used. To study the effect of KCl on Bti toxin hemolysis, a kinetic hemolytic bioassay at a toxin concentration of 2 nM was carried out in 20 mM Tris 150 mM KCl pH 7.4 and compared with a standard 20 mM Tris 150 mM NaCl pH 7.4 bioassay. Depending on the amount of hemolysis (as compared to the control) the results in Table 11 are classified as to no effect (85-100%), some effect (50-84%), and pronounced effect (0-49%). DTT (75 mM), EDTA (50 mM), EGTA (100 mM), ATP (25 mM), and BSA (150 uM) all had no effect; $MgCl_2$ (250 mM) did have some effect on hemolysis (59%). Lowering magnesium to 100 mM gave 100% hemolysis (data not shown). Sucrose (570 uM) also showed some effect (83%) on hemolysis. Lowering the sucrose concentration to 380 uM gave 100% hemolysis (not shown). Changing the salt in the buffer to 150 mM KCl from 150 mM NaCl also had no effect on hemolysis as the kinetic hemolytic bioassay curves were similar in shape (data not shown).

TABLE 9

Substances Examined for Inhibition of Bti Toxin Hemolysis

Substance	Concentration	Toxin concentration (nM) for bioassay	preincubation for 15 minutes
DTT	75 mM	16	+
EDTA	50 mM	8	+
EGTA	100 mM	8	+
ATP	25 mM	8	+
$MgCl_2$	250 mM	2	
Sucrose	570 uM	16	
BSA	150 uM	16	
MAB	1/10 dil'n	8	*

* MAB preincubated with toxin for 30 minutes.

Effect of Ion Channel and Ion Pump Antagonists on Bti Toxin Hemolysis

To determine if the Bti toxin was interacting with a specific ion channel or pump in the erythrocyte membrane, a series of compounds reported to be ion channel or ion pump antagonists were preincubated with erythrocytes for 15 minutes at 37°C followed by a fixed-time hemolytic bioassay at a toxin concentration of 8 nM. Table 10 lists the type of channel/pump each agent inhibited, the concentrations employed, the minimal effective concentration for 90% inhibition, and the type of cell that the minimum effective concentration was determined on. Table 11 shows that the ion channel/pump antagonists, TEA, verapamil, DIDS, tetrodotoxin, and ouabain had no effect on hemolysis. Furosemide did show some effect (66%) at 7.5 mM. Lowering the furosemide concentration to 3.75 mM gave 81% hemolysis; at 1 mM, (concentration of 90% inhibition), 100% hemolysis was observed (not shown).

TABLE 10

Ion Channel And Pump Antagonists

Agent	Channel	Pump	Concentration employed	effective concentration	cell type	reference
TEA(*)	K^+		100 mM	1 mM	squid giant axon	1
verapamil	Ca^{++}		100 uM	1 uM	smooth muscle rabbit aorta	2
Tetrodotoxin	Na^+		63 uM	1 uM	lobster giant axon	4
DIDS(**)	Anion		1 mM	2 uM	erythrocyte	3
Furosemide	Anion(and) Na^+/K^+		7.5 mM	1 mM	erythrocyte	5,6
Ouabain		Na^+/K^+ ATPase	1 mM	10 uM	erythrocyte	7

* TEA: tetraethylammonium
**DIDS: 4,4' Diisothiocyano-2,2'-stilbene disulfonic acid

1. Armstrong 1966
2. Terai et al. 1981
3. Narahashi et al. 1964
4. Cabantchik and Rothstein 1974
5. Brazy and Gunn 1976
6. Garay et al. 1981
7. Post et al. 1960

Effect of Purified Monoclonal Antibody on Bti Toxin Hemolysis

The effect of the purified monoclonal antibody (MAB) on Bti toxin hemolysis was studied to determine if the binding of antibody to the antigenic site of the Bti toxin could affect cytotoxic activity. The purified MAB was used because in inhibition studies carried out with the MAB described in Armstrong et al. (1985), a reduction of hemolytic activity was seen However, incubation of toxin with the control ascites fluid also gave reduced hemolysis. This suggested that the inhibition of hemolytic activity seen with the MAB of Armstrong et al. (1985) was non-specific, due to components in the ascites fluid. The toxin was incubated with the purified MAB in a 1/10 dilution for 30 minutes at 37°C prior to the start of a fixed-time hemolytic bioassay (Table 9). The MAB up to a dilution of 1/10 did not have any effect on hemolysis (Table 11). The control ascites fluid purified in the same way as the MAB, did not give any inhibition of hemolysis. Thus, the inhibition of Bti toxin hemolysis seen with the unpurified MAB prepared by Armstrong et al. (1985) was a non-specific interaction and the purified MAB was non-neutralizing for toxin hemolytic activity. To insure that the purification process did not inactivate the antibody, an ELISA of the purified MAB performed according to Armstrong et al. (1985) was positive down to a 1/4000 dilution.

Effect of ATP Depletion and Neuraminidase Treatment of Erythrocytes on Bti Toxin Hemolysis

In order to determine if Bti toxin hemolysis required ATP for enzymatic action or for interaction with an energy-requiring ion pump, a kinetic hemolytic bioassay was performed with ATP-depleted cells at a toxin concentration of 8 nM. The curves for the ATP-depleted cells and the control (fresh) cells were similar in shape, which showed that ATP depletion did not affect the ability of the toxin to lyse the cells (data not shown). In order to investigate

whether the Bti toxin required association with an erythrocyte membrane receptor containing a terminal sialic acid group, a fixed-time hemolytic bioassay was run with neuraminidase-treated erythrocytes at a toxin concentration of 8 nM. Table 11 shows that the there was no inhibition of hemolysis by the neuraminidase treatment.

Responsiveness of Chicken Erythrocytes to Bti Toxin

To determine the ability of the Bti toxin to hemolyze nucleated erythrocytes, a fixed-time hemolytic bioassay was carried out with 1 X chicken and human erythrocytes at toxin concentrations of 2, 4, 8, 32, and 80 nM. Chicken erythrocytes were not as responsive to toxin hemolysis as human cells (Figure 19). At a large excess of toxin (80 nM), the responsiveness was the same, but at the lower concentrations of toxin (4 and 8 nM), the chicken cells were, respectively, 56% and 68% (Table 11) as responsive.

TABLE 11

Effect of Substances and Treatments on Bti Toxin Hemolysis

Treatment	No effect 85-100%	Some effect 50-84%	Pronounced effect 0-49%	Toxin Conc. nM in Bio-assay
DTT	75 mM (100%)			16
EDTA	50 mM (92%)			8*
EGTA	100 mM (92%)			8*
ATP	25 mM (99%)			8*
KCl	150 mM (94%)			2
Mg^{++}		250 mM (59%)		8
Ca^{++}			10 mM (21%)	8[1]
TEA	100 mM (100%)			8*
Verapamil	100 uM (94%)			8*
DIDS	1 mM (100%)			8*
Tetrodotoxin	63 uM (100%)			8*
Furosemide		7.5 mM (66%)		8*
Ouabain	1 mM (100%)			8*
Sucrose		570 mM (83%)		16
BSA	150 uM (85%)			16
MAB	1:10 dil'n (91%)			8
ATP-depleted cells	(100%)			8
Neuraminidase-treated cells	(100%)			8
Chicken Erythrocytes		(68%)		8

$$\% \text{ Hemolysis} = \frac{\% \text{ Hemolysis treatment}}{\% \text{ Hemolysis no treatment}} \times (100)$$

* = Preincubation for 15 minutes
[1]see Fig 17

FITC Labelling of Bti Toxin and Fluorescence Binding Study

Bti toxin was labelled with FITC and 97% of the protein was recovered in the labelling reaction and it was hemolytically active (Table 12). Labelling efficiency was low as only 1 out of every 6 toxin molecules were labelled (each toxin molecule contains 10 lysines; Armstrong et al. 1985). In order to visualize any binding of Bti toxin to the erythrocyte membrane, a kinetic hemolytic bioassay was performed using FITC toxin at 8 nM. At the time intervals 0, 10, 20, 30, 40, 50, and 60 minutes, aliquots were removed and layered over a Ficoll solution (Histopaque-Sigma) diluted 1/1 with T/N buffer. The cells were then centrifuged for 2 minutes at 3350 x g, and the pellet was examined for binding under a Zeiss fluorescent microscope. Fluorescence microscopy did not show any association of the toxin with the cell in the early part of the bioassay (during the lag period). Fluorescence was seen at 20 minutes (60% hemolysis). However, the binding appeared to be to cells that had already lysed.

TABLE 12

FITC Labelling of Bti Toxin

% Recovery of protein	97%
FITC/lysines in labelling reaction	3.6
Number of FITC'S/toxin	1/6

Effect of Temperature on Bti Toxin Biotoxicity on *Aedes* Insect Cells

To determine the effect of temperature on Bti toxin toxicity as measured by the *Aedes* insect cell bioassay, toxin was heated in separate tubes at 400 nM in 10 mM phosphate buffer 7.4 (P-10) for 50 minutes at 40, 44, 46, 48, and 50°C. *Aedes* insect cell bioassays

were then performed at 30 nM toxin concentration. There was a sharp decrease in biological activity between 48 and 50°C (Figure 20). Thus, 50 minutes for 50° was chosen as a standard condition for Bti toxin heat inactivation. In addition, quantitative *Aedes* insect cell bioassays have shown the toxin to be between 45 and 65% inactivated under these conditions.

Effect of NaCl on the Heat Inactivation of Bti Toxin

Bti toxin (15 uM) was heated at 50°C for 50 minutes in P-10 buffer in the presence of 100 and 500 mM NaCl and then assayed for *Aedes* insect cell biotoxicity at 30 nM. The toxin (6.3 uM) was also was heated at 50°, 60°, and 70°C for 50 minutes in P-10 buffer in the presence of 200 and 500 mM NaCl and assayed for *Aedes* insect cell biotoxicity at 40 nM. All heat-treated toxins were compared to the control toxin incubated at 23°C. Table 13 shows that NaCl protected Bti toxin from standard condition (50°C) heat inactivation. However, this protection was lost at temperatures of 60°C and higher.

TABLE 13

Effect of NaCl on Heat Inactivation of Bti Toxin as Determined by *Aedes* Insect Cell Bioassay

	NaCl (mM)			
Temperature	0	100	200	500
50°C	-	+	+	+
60°C	-		-	-
70°C	-		-	-

\+ biologically active

\- biologically inactive

Protein Concentration, Dye Binding and Polyacrylamide Gel Migration of Heat-Treated Bti Toxin

In order to determine if there was loss of protein in solution upon heating, Bti toxin (17 uM) was heat-treated in P-10 buffer for 50°C for 50 minutes, then 8 ug of heat-treated and control toxin (23°C x 50 minutes) were assayed for protein concentration by the method of Lowry as modified by Peterson (1983). The Lowry protein assay showed the same protein concentration for heated and unheated toxin (Table 15). To determine if heating the toxin caused structural changes, such that it would bind a different amount of dye as compared to the native toxin, Bti toxin was heated as above for the Lowry assay and analyzed for binding of Coomassie brilliant blue. There was about 2.5 times more binding of dye for the heat-denatured as opposed to the unheated control (Table 15). To determine if the heat-treated toxin had an altered structure such that its charge or molecular weight was changed, Bti toxin heated at 50°C for 50 minutes was subjected to native polyacrylamide gel electrophoresis. The heat-treated toxin showed no migration as it was unable to enter the gel (Table 15). Normal migration was seen for the unheated control toxin. This suggested that the heat-inactivated toxin was in an aggregated or oligomeric form too large to enter the gel or that the toxin had its negative charge eliminated such that it did not migrate into the electric field.

Effect of Heat on Bti Toxin Intrinsic Fluorescence and *Aedes* Insect Cell Biological Activity

To examine the effect of heating Bti toxin on intrinsic fluorescence and *Aedes* insect cell biological activity, toxin 400 nM in P-10 buffer was heated at 50°C for 50 minutes. The control toxin was likewise incubated at 25°C. After the incubation, the intrinsic fluorescence spectra of each were measured and an *Aedes* insect cell bioassay was carried out at 30 nM. Figure 21 shows a 20% reduction

in fluorescence intensity, no change in the maximal wavelength of 335 nm and loss of biological activity. There was no recovery of any of these parameters following cooling.

Effect of Heat on Bti Toxin Hemolysis

To study the effect of heat on the Bti toxin erythrocyte interaction, toxin (17 uM) was heat-treated at 50°C in P-10 buffer for 0, 15, 30, 40, 50, and 60 minutes. For each time interval, a fixed-time hemolytic bioassay was done at a toxin concentration of 8 nM. Control toxin was incubated at the same time intervals at 37°C. Figure 22 shows that a 60 minute heat treatment inactivated the toxin to about 10% of the control. The heat inactivation curve was biphasic: a 50% loss of activity from 15 to 30 minutes, followed by a plateau from 30 to 50 minutes, and from 50 to 60 minutes, an additional loss of activity to 10% hemolysis.

Reversibility of Partially Heat-Inactivated Bti Toxin on Hemolysis

To determine the reversibility of a partially heat-inactivated Bti toxin, toxin (17 uM) in P-10 buffer was incubated for 40 minutes at 50°C and then cooled to 25°C. A fixed-time hemolytic bioassay (toxin concentration 8 nM) was then done immediately and then at 1, 2, and 4 hours. The control toxin was incubated at 25°C over the same interval. Incubation of the heat-denatured Bti toxin at 25°C for up to 4 hours failed to restore biological activity (Table 14). In fact, there appeared to be a further loss of biological activity during the 4 hours after heat removal (Table 14).

TABLE 14

Irreversible Heat Denaturation of Bti Toxin

Incubation time (hr)	Hemolysis % of control
0	61
1	27
2	9
4	14

TABLE 15

Bti Toxin Denaturation and Renaturation

Heat and Urea

	Urea	Heat
	6 M x 15 hours then dilute to 0.5 M	50°C x 50 minutes then cool to 23°C
Fluorescence		
Maximal Wavelength (nm)		
native	334	335
denatured	337	335
renatured	335	335
Intensity[1]		
denatured	59	80
renatured	85	80
Aedes cell insect bioassay		
denatured	no activity	no activity
renatured	no activity	no activity
Dye binding[2]		
Coomassie Blue	3.0	2.5
Lowry Protein Assay[2]	---	1.0
PAGE migration		
denaturing	migration	---
non-denaturing	no migration	no migration

[1] percent of the native toxin
[2] denatured toxin/native toxin
-- not done

Effect of Urea on Bti Toxin Intrinsic Fluorescence and *Aedes* Insect Cell Biological Activity

To study the effect of the denaturant, urea, on Bti toxin intrinsic fluorescence and *Aedes* insect cell biological activity, Bti toxin (9.6 uM) was incubated in 6 M urea 150 mM NaCl 10 mM phosphate buffer pH 7.4 at 23°C for 15 hours. Control Bti toxin was also incubated under the same conditions except that the urea was omitted. After 15 hours incubation, intrinsic fluorescence spectra were taken. In addition, *Aedes insect* cell bioassays at a toxin concentration of 8 nM were performed. There was a 41% drop in fluorescence intensity and a 3 nm red shift in the wavelength (334 to 337 nm) after urea treatment (Figure 23 and Table 15). Biological activity was 95% inactivated, as an *Aedes* insect cell bioassay of up 20 times urea-treated toxin, as compared to control toxin, failed to show any cytolysis (Table 15). All fluorescence spectra were corrected for the fluorescence due to the buffer with and without urea. Dilutions for the *Aedes* insect cell bioassay were such that the cells were not exposed to more than 1 M urea and showed no signs of damage. Exposure of toxin to 1 M urea did not affect its ability to lyse cells.

Reversibility of Urea Denaturation of Bti Toxin

To ascertain whether the urea denaturation was reversible, both native and denatured toxin treated above were diluted 12-fold with phosphate NaCl buffer to a urea concentration of 0.5 M and toxin concentration of 800 nM and were incubated at 23°C for 2 hours. Fluorescence spectra and *Aedes* insect cell bioassays were then performed as described. Figure 23 shows an increase in intensity upon removal of the urea (by dilution) to 85% of the control and almost a complete recovery of the wavelength peak to 335 nm (native toxin initially was 334 nm; Table 15). Biological activity was not restored as the urea-renatured Bti toxin was still 95% inactivated

towards *Aedes* insect cells.

In an effort to demonstrate reactivation of the urea-denatured toxin, other methods allowing a slower renaturation were employed. Bti toxin was denatured in 6 M urea in a similar experiment as above, and was renatured by dialysis for 6 hours and examined up to 21 hours for fluorescence and *Aedes* insect cell biotoxicity. In another experiment, toxin was denatured in 6 M urea and renatured by stepwise dilution 6 M - 4 M - 2 M - 0.5 M (two hours at each concentration) and examined up to 20 hours as above. These experiments gave similar results with regard to fluorescence intensity, maximal wavelength, and biological activity as the direct dilution experiment above.

Dye Binding and Polyacrylamide Gel Migration of Urea-Denatured Bti Toxin

The urea-denatured toxin could have undergone a structural change such that it bound a different amount of dye than the native protein. 4 ug of native and 6 M urea-treated toxin were subjected to the Bio-Rad protein assay, for a determination of Coomassie brilliant blue absorbance. The dye-binding data showed that the urea-treated denatured toxin bound about 3 times more Coomassie blue than native toxin (Table 15). Similar to heat denaturation, the urea-denatured toxin could also have had an aggregated structure or charge, such that its mobility in a polyacrylamide gel was different from than the native toxin. Urea-denatured toxin was subjected to native polyacrylamide electrophoresis and no migration was seen. To determine if this denatured structure was susceptible to SDS, the urea-denatured toxin was also analyzed on a denaturing gel and normal migration was seen (Table 15).

Effect of Biotinylation of Bti Toxin on Hemolysis

As another way of studying the interaction of the Bti toxin with the erythrocyte membrane, toxin was labeled with biotin, which covalently links to lysines, for use in a binding study using the avidin-biotin technique described by Fucillo (1985). This method consisted of first conjugating biotin to Bti toxin, incubating the biotinylated toxin with erythrocytes and then visualizing toxin binding by adding FITC-avidin.

In order to determine the effect of the time of biotinylation on hemolytic activity, at 5 minutes, 1, 2, 3, and 4 hours, aliquots were withdrawn from a biotinylation reaction (at 25°C) of the Bti toxin and fixed-time hemolytic bioassays at a toxin concentration of 8 nM were done. The control toxin was treated as above, except without the biotin reagent. Biotinylation of the Bti toxin caused a drastic inactivation of hemolytic activity. After only 5 minutes of biotinylation, doubling the toxin concentration resulted in only 8% hemolysis (Table 16). This indicated that more than half of the toxin molecules had been inactivated during the 5 minute course of the labelling reaction. After 3 hours of biotinylation, the toxin gave no hemolysis. The control toxin gave 100% homolysis up to 4 hours. Due to this loss of activity, the biotinylated toxin was not suitable for further binding studies with FITC-avidin. However, the results of the biotinylation studied implied that lysines were essential for biologic activity of the Bti toxin.

TABLE 16

Effect of Biotinylation of Bti Toxin on Hemolytic Activity

Time of Biotinylation	Toxin Concentration nM	% Hemolysis
5 minutes	8	0
	16	8
	80	100
1 hr	8	3
	16	3
	80	48
2 hr	8	2
3 hr	8	0
4 hr	8	0

Phospholipase C Determination

Bti toxin was assayed for phospholipase C activity as other hemolytic and cytolytic bacterial toxins have been shown to have this activity (Stephen and Pietrowski 1981). The assay relied on the increase in turbidity of an egg yolk extract measured at 540 nm as the micelles in the yolk break up due to the hydrolysis of phosphatidyl choline to its diglyceride and phosphoryl choline. Table 17 shows that the Bti toxin showed no turbidity up to 160 nM (40 times the amount needed to lyse erythrocytes). The *C. perfringens* phospholipase C control gave a large increase in turbidity. The 48.6 and 97.2 milliunits/ml assays were very turbid such that they had to be diluted 10 times to give an OD_{540} of less than 2.0.

TABLE 17

PHOSPHOLIPASE C DETERMINATION

Egg Yolk Turbidimetry

C. perfringens	no dilution	1:10
milliunits/ml(1)	A_{540}	
9.72	4.30	0
48.60	9.37	0.974
97.20	17.97	1.720
Bti Toxin (nM)		
16	0	0
160	0	0

Incubation 37°C x 60 minutes.

(1) 1 unit will liberate 1.0 umole of water soluble organic phosphorous from L-alpha phosphatidyl choline per minute, pH 7.3 at 37°C.

Spectroscopic Determination of Tryptophan and Tyrosine

Knowledge of the tryptophan content of the Bti toxin is important in planning and interpreting biophysical fluorescence studies. Tryptophan, in a protein, has the highest quantum yield out of all the aromatic amino acids, and it is the major contributor to the intrinsic fluorescence (Longworth 1971). A spectrophotometric determination of the tryptophan content of the Bti toxin was done because the amino acid analysis performed by Armstrong et al. (1985) did not include an analysis of tryptophan.

Davidson and Yamamoto (1984) reported an amino acid analysis of the 25 Kd purified Bti toxin, but did not analyze for tryptophan. In

addition to tryptophan, the Edelhoch (1967) method also yielded the number of tyrosines. The 8.3 tyrosines per Bti toxin molecule determined by the method of Edelhoch (1967) method agreed well with the 8.0 tyrosines determined by amino acid analysis by Armstrong et al. (1985) and was also close to 7 tyrosines determined for the 25 Kd protein of Davidson and Yamamoto (1984) (Table 18). Waalwijk et al. (1985) have cloned the Bti toxin gene for the 28 Kd protein and determined 3.0 tryptophans and 7.0 tyrosines per molecule. However, when account is taken for the loss of the first 29 residues of Waalwijk et al's. (1985) sequence (Appendix B), due to the protein of Armstrong et al. (1985) being smaller, then there were only 2 tryptophans (Table 18). The difference between the 2.9 tryptophans determined for the protein of Armstrong et al. (1985) and that of 2.0 determined from the nucleotide sequence of Waalwijk et al. (1985) could be due to the fact that there were strain differences between the bacteria being used by the two laboratories that served as a source of the Bti toxin. Armstrong et al. (1985) published the sequence (determined on a protein sequenator) of the first 30 amino acids from the N terminal end of the toxin molecule. This was identical to a portion of the N terminal sequence of the 28 Kd protein of Waalwijk et al. (1985) except that Waalwijk et al. (1985) found a methionine where Armstrong et al. (1985) found a serine. If there was already reported one difference in these 30 amino acids between the two proteins, it was possible there were other differences in the rest of the protein, and this could have given rise to a difference in tryptophan content. The tryptophan plus tyrosine determination of lysozyme and RNAse A (Table 18) were very close to the results reported by Edelhoch (1967), thus adding reliability to the values determined for the Bti toxin.

TABLE 18

SPECTROSCOPIC DETERMINATION OF TRYPTOPHAN AND TYROSINE

	trp	tyr	reference
Bti toxin			
	2.9	8.3	(1)
	---	8.0	(2)
	2.0	7.0	(3)
		7.0	(4)
Lysozyme			
Experiment I	5.4	2.5	(1)
Experiment II	5.1	2.7	(1)
	6	3	(5)
RNAse			
	0.2	5.2	(1)
	0	6	(5)

(1) determined as described by Edelhoch (1967)
(2) Armstrong et al. (1985)
(3) Waalwijk et al. (1985)
(4) Davidson and Yamamoto (1985)
(5) determined by amino acid analysis by Edelhoch (1967)

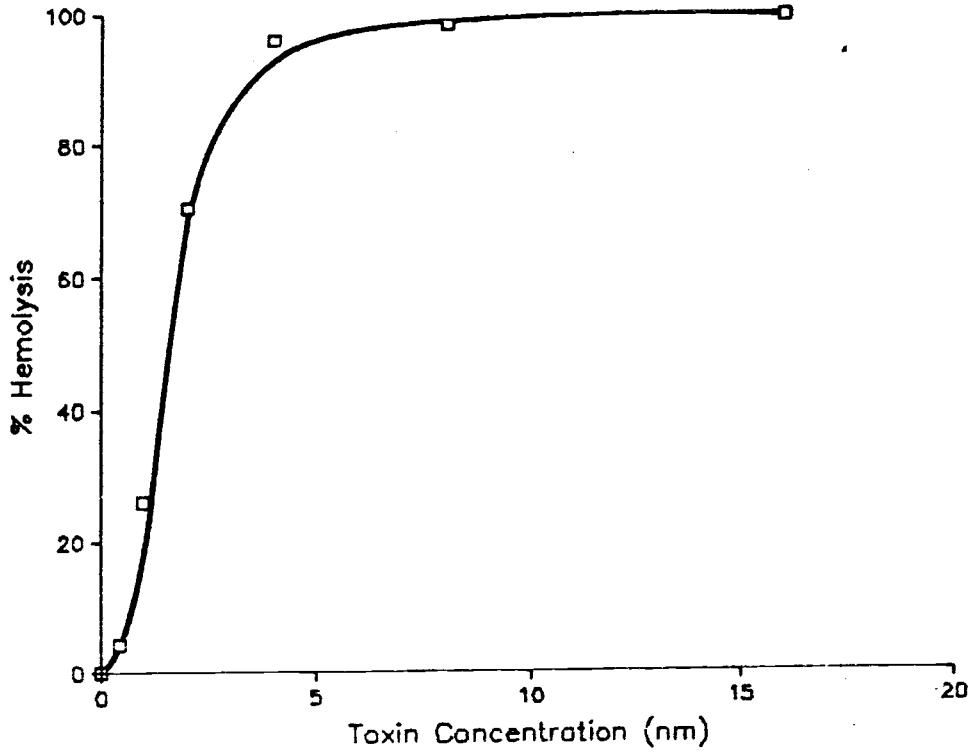

Figure 3. Standard end point hemolytic bioassay for Bti toxin.

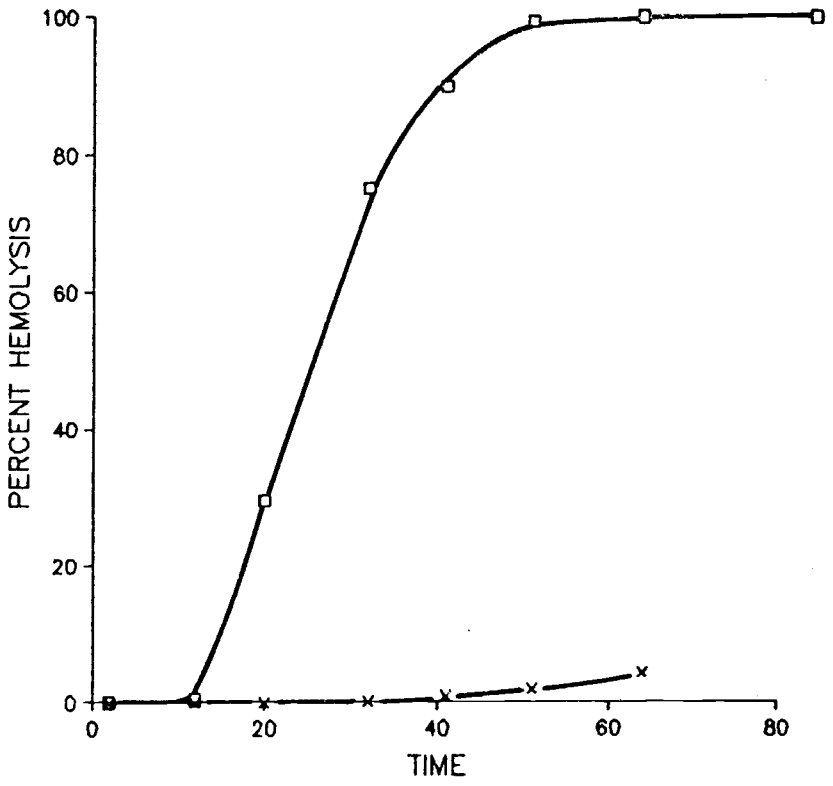

Figure 4. Standard kinetic hemolytic bioassay for Bti toxin. 4 nM (□) 0.44 nM (X)

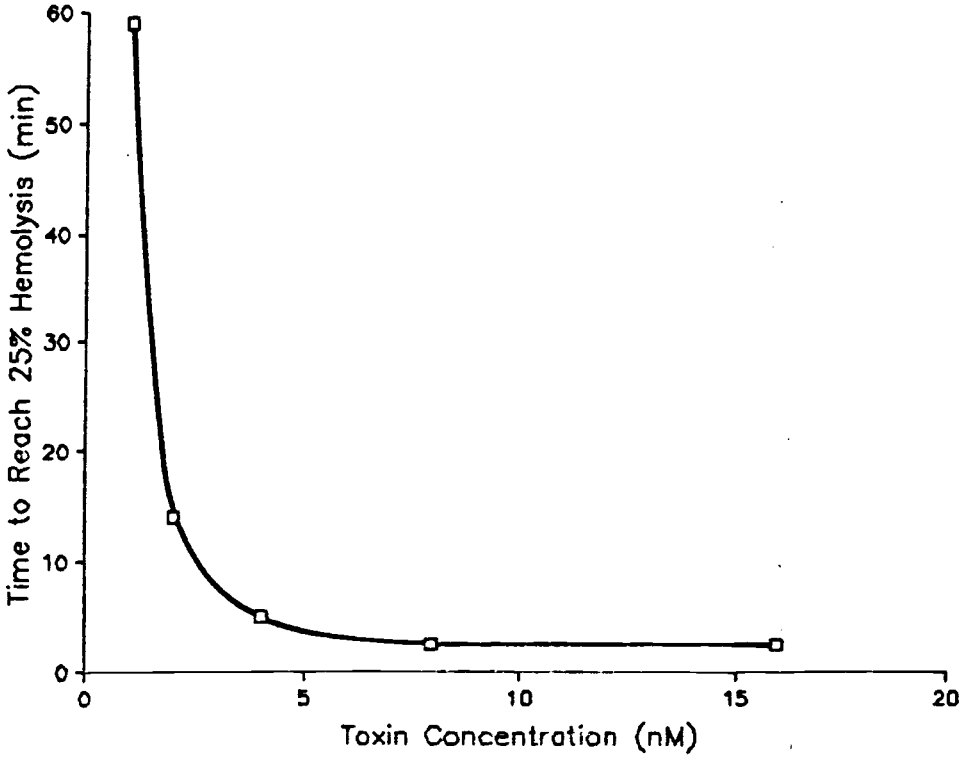

Figure 5. Effect of Bti toxin concentration on the time (minutes) to reach 25% hemolysis.

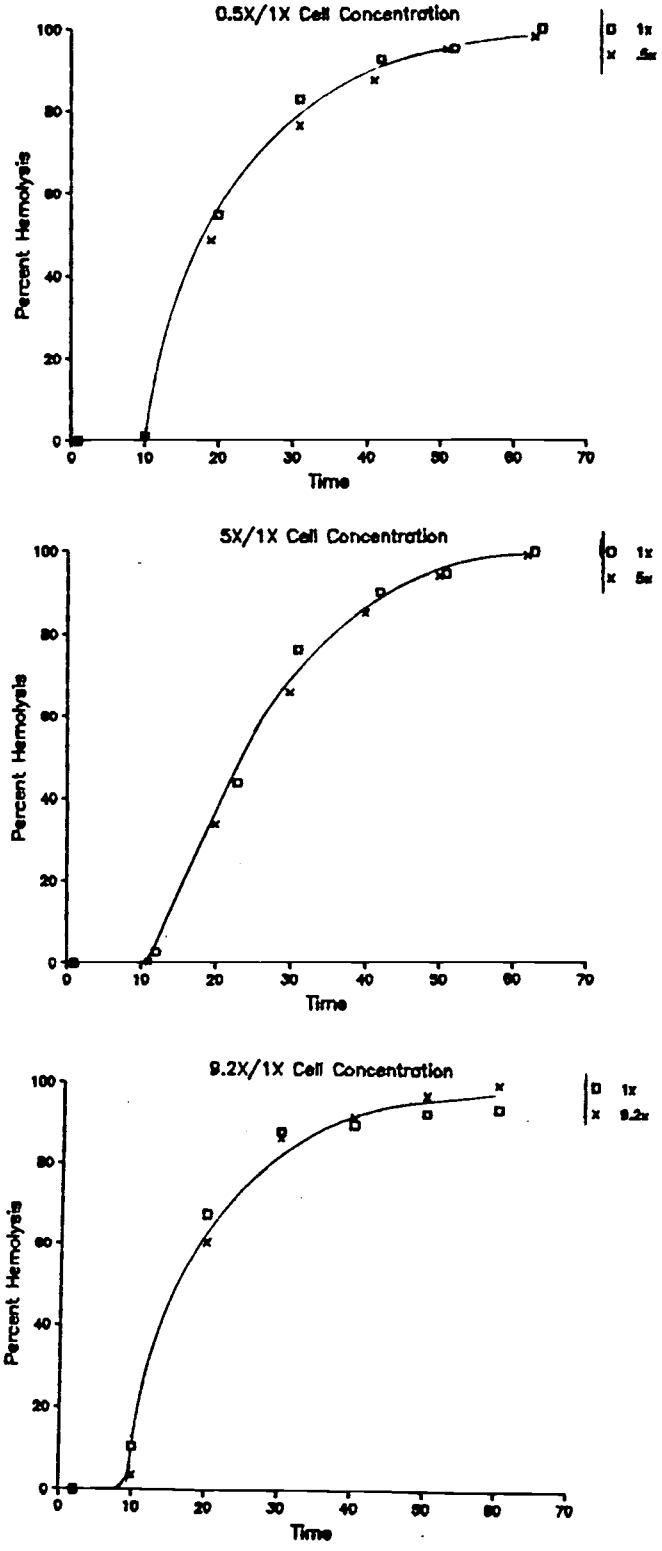

Figure 6. Effect of cell concentration on the kinetics of Bti toxin hemolysis.
1X = 2.5 10^7 cells/ml

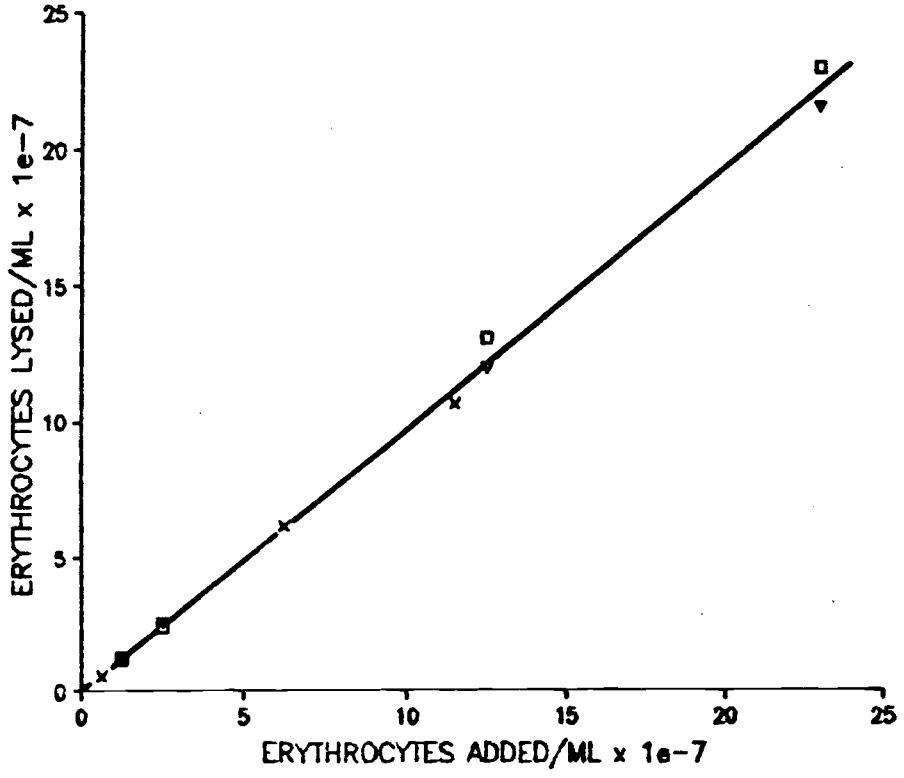

Figure 7. Effect of cell concentration on the extent of Bti toxin hemolysis.

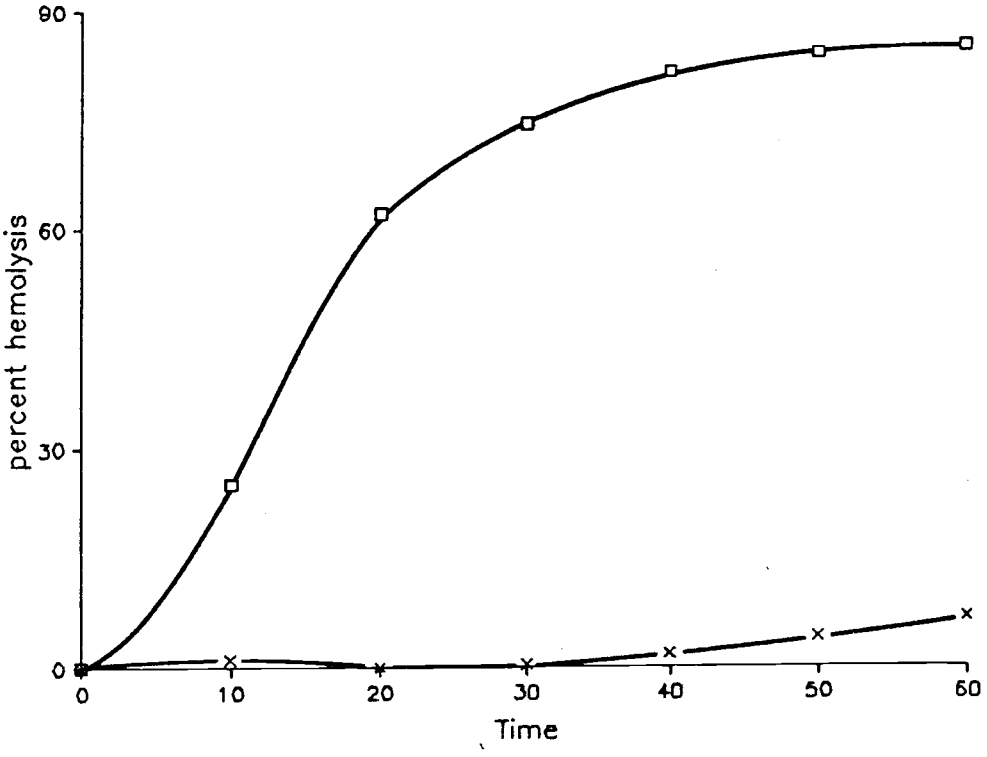

Figure 8. Effect of pH 5 (X) as compared to pH 7 (□) on Bti toxin hemolysis.

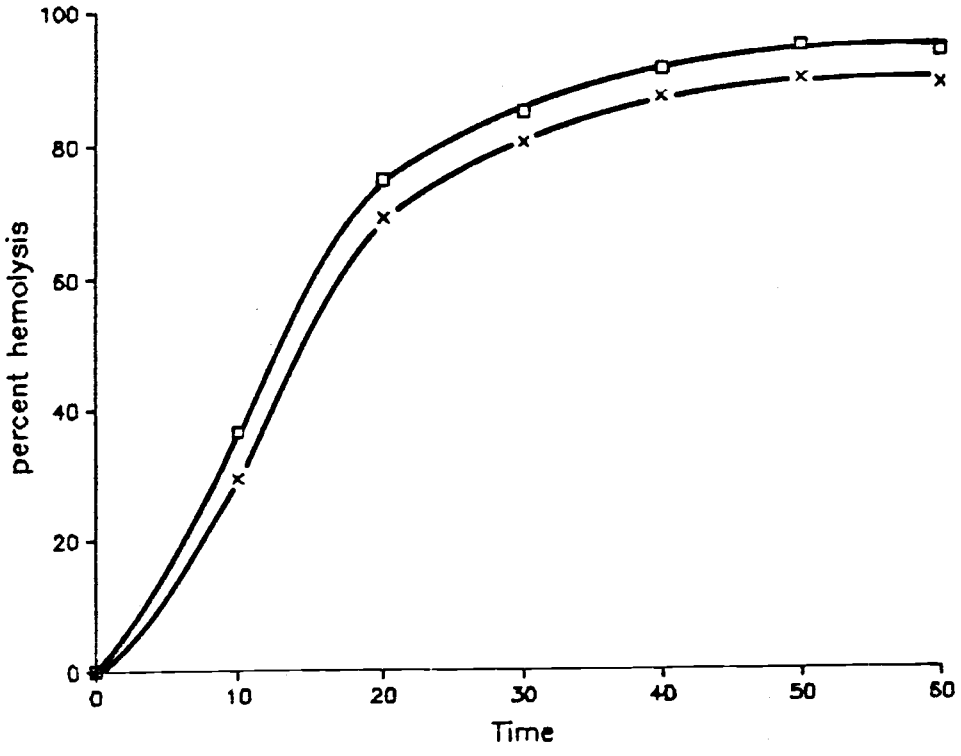

Figure 9. Effect of pH 6 (X) as compared to pH 7 (□) on Bti toxin hemolysis.

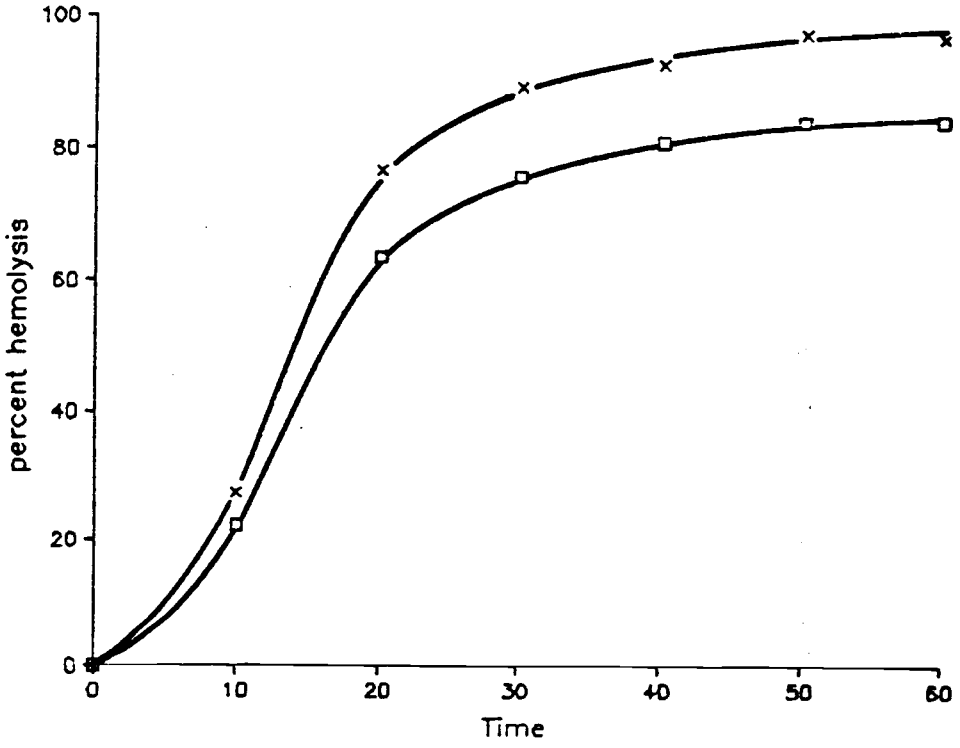

Figure 10. Effect of pH 8 (□) as compared to pH 7 (X) on Bti toxin hemolysis.

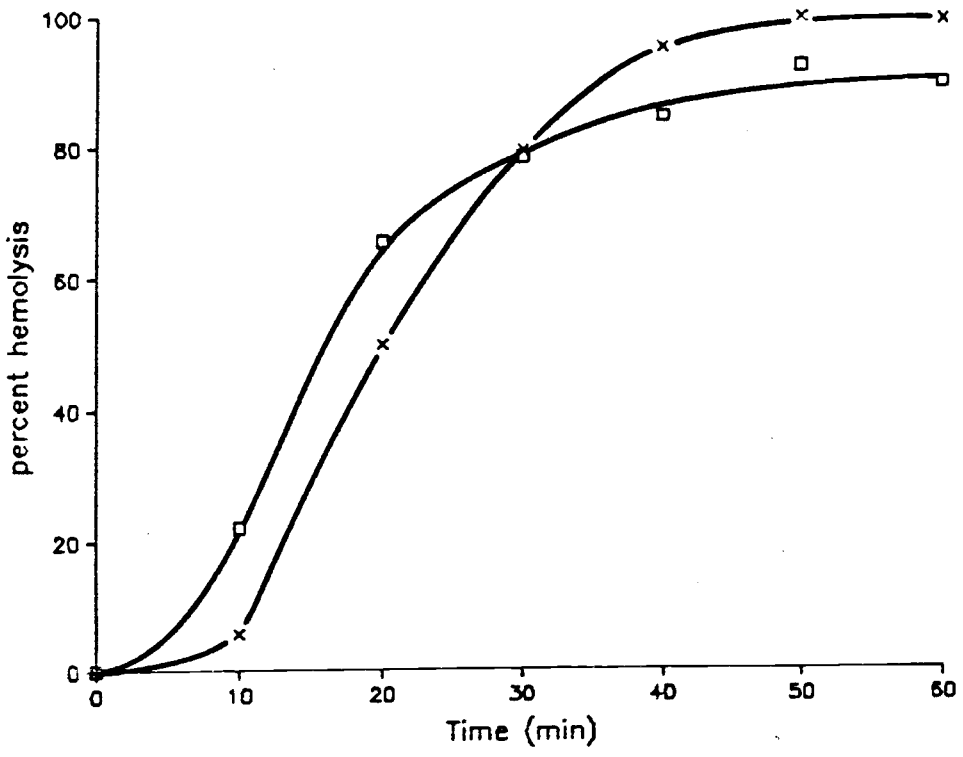

Figure 11. Effect of pH 9 (□) as compared to pH 7 (X) on Bti toxin hemolysis.

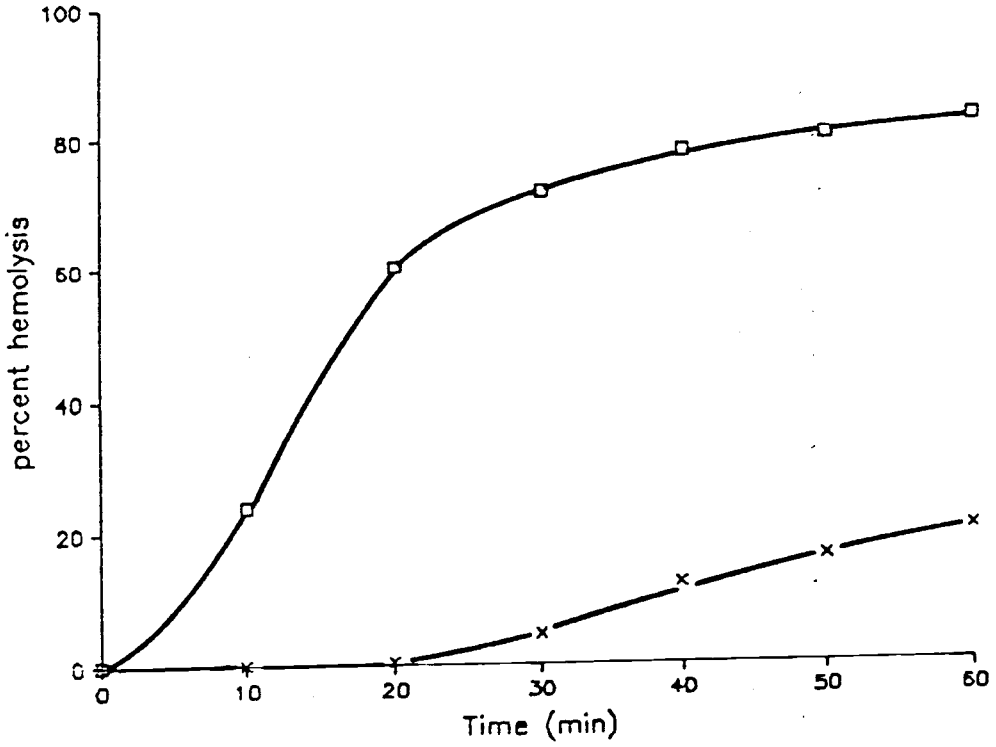

Figure 12. Effect of pH 10 (X) as compared to pH 7 (□) on Bti toxin hemolysis.

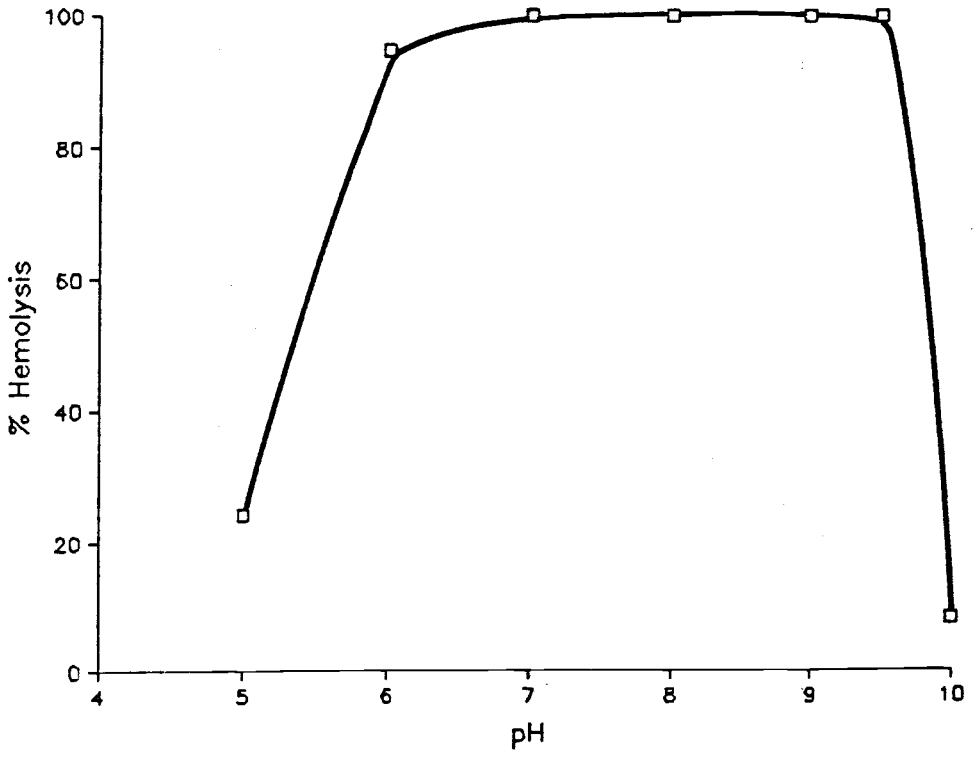

Figure 13. Effect of pH on Bti toxin hemolysis.

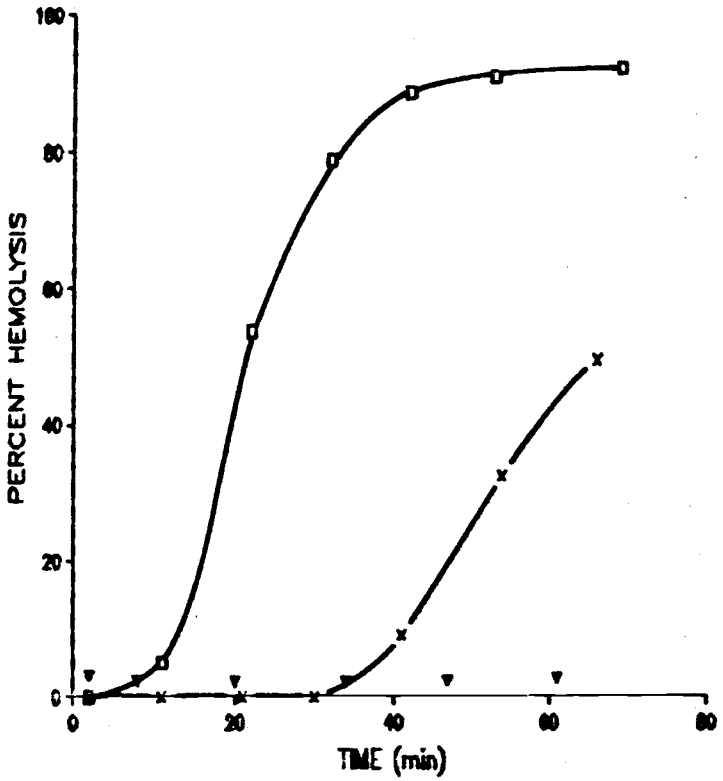

Figure 14. Effect of temperature on Bti toxin hemolysis. 37° (□), 20° (X), and 0°C (▽).

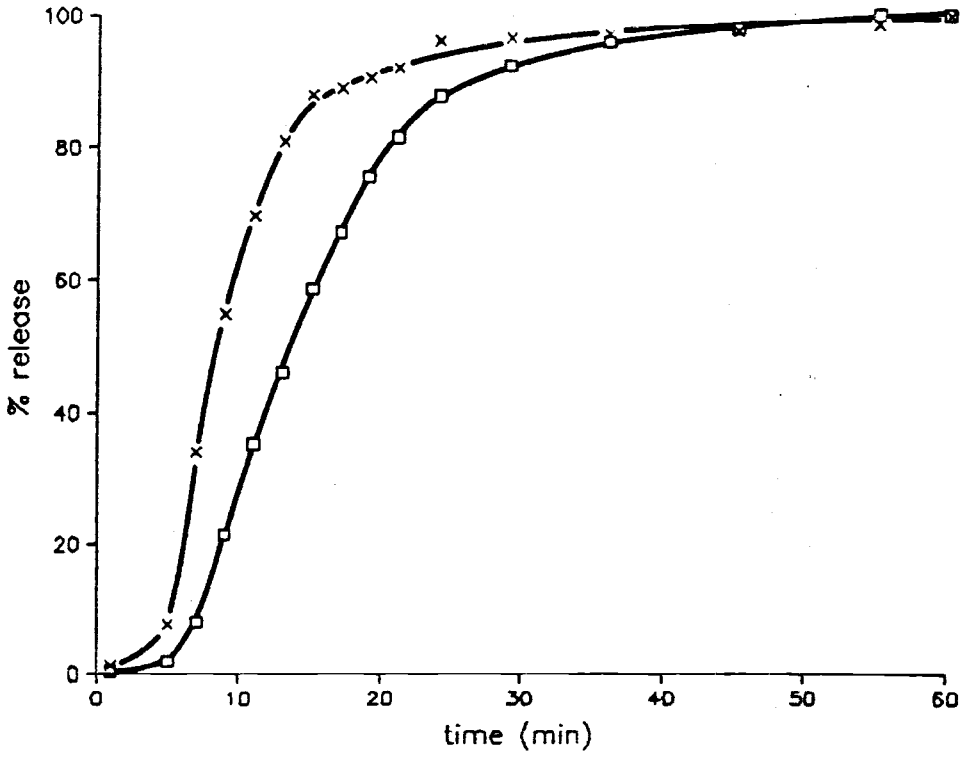

Figure 15. Relationship of potassium release to hemoglobin release at 37°C. Potassium (X) Hemoglobin (□)

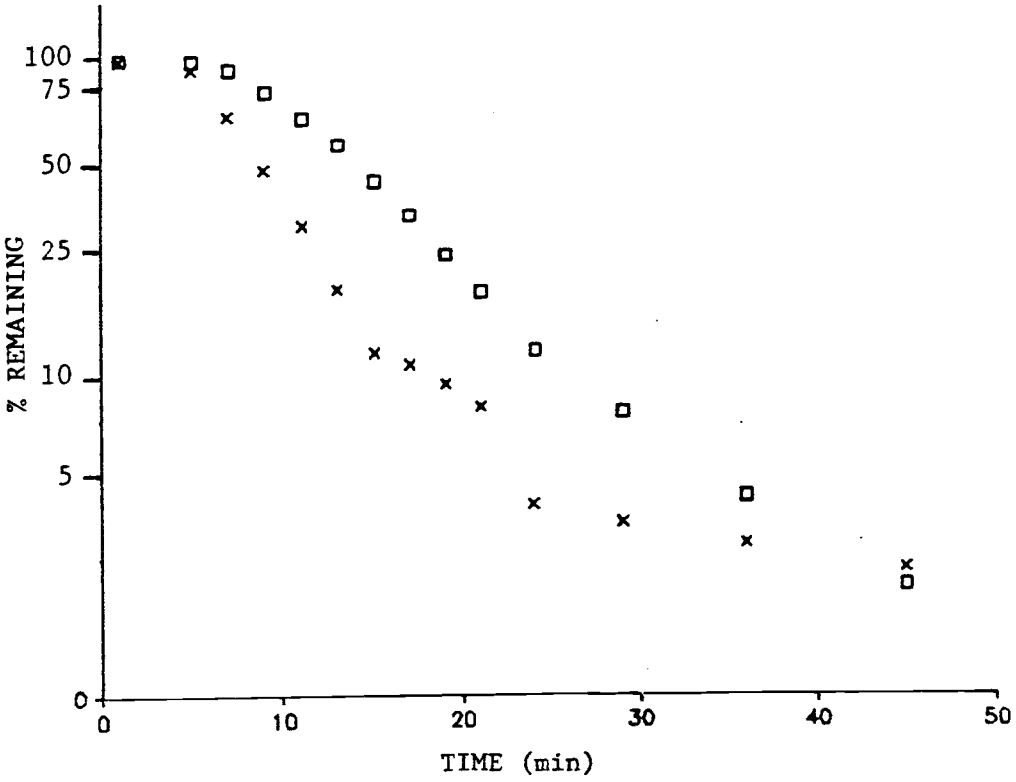

Figure 16. First order rate plot of hemoglobin and potassium release. Potassium (X) Hemoglobin (□)

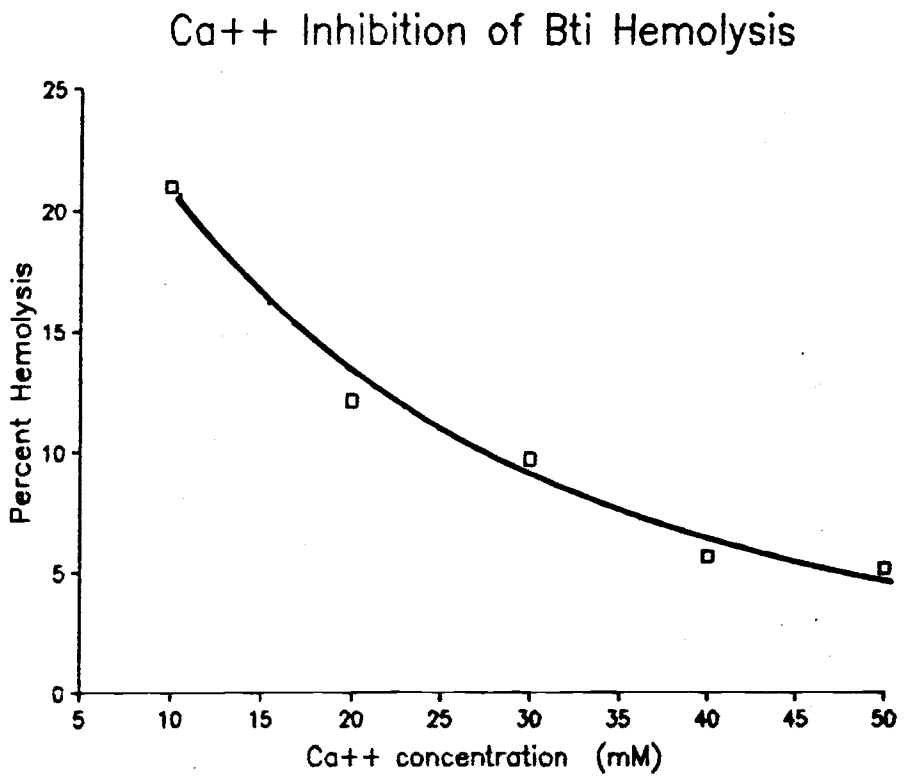

Figure 17. Effect of calcium on Bti toxin hemolysis.

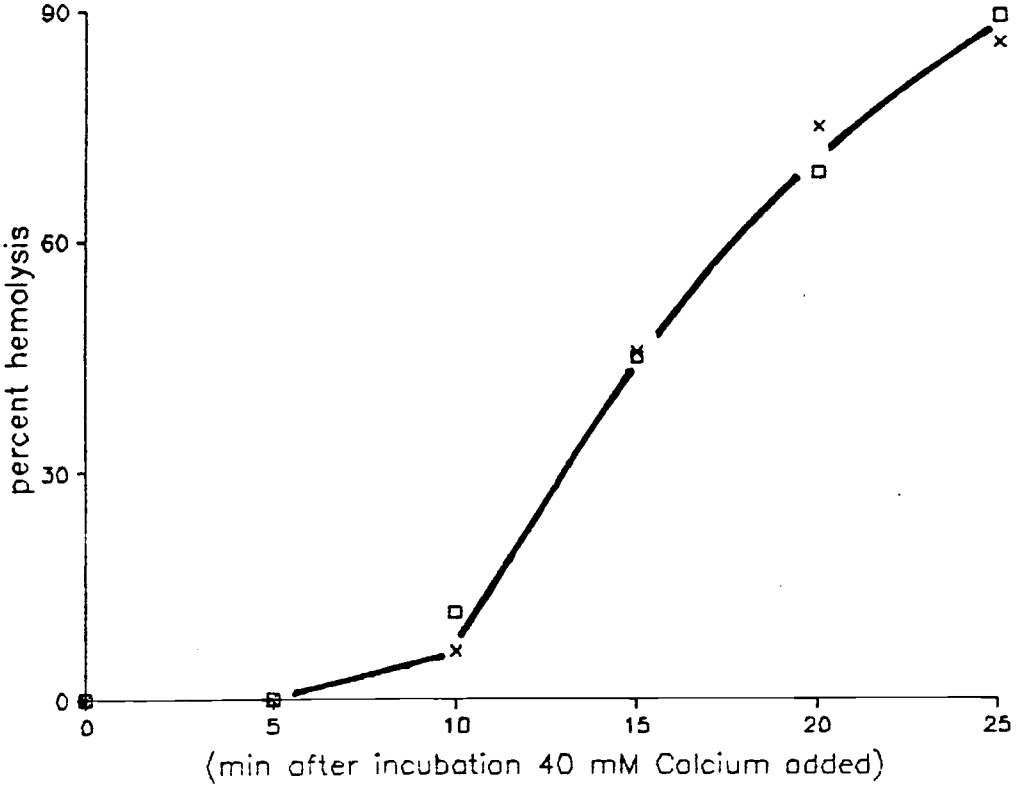

Figure 18. Effect of 40 mM calcium in inhibiting Bti toxin hemolysis during the assay. No Calcium (X) Calcium (□)

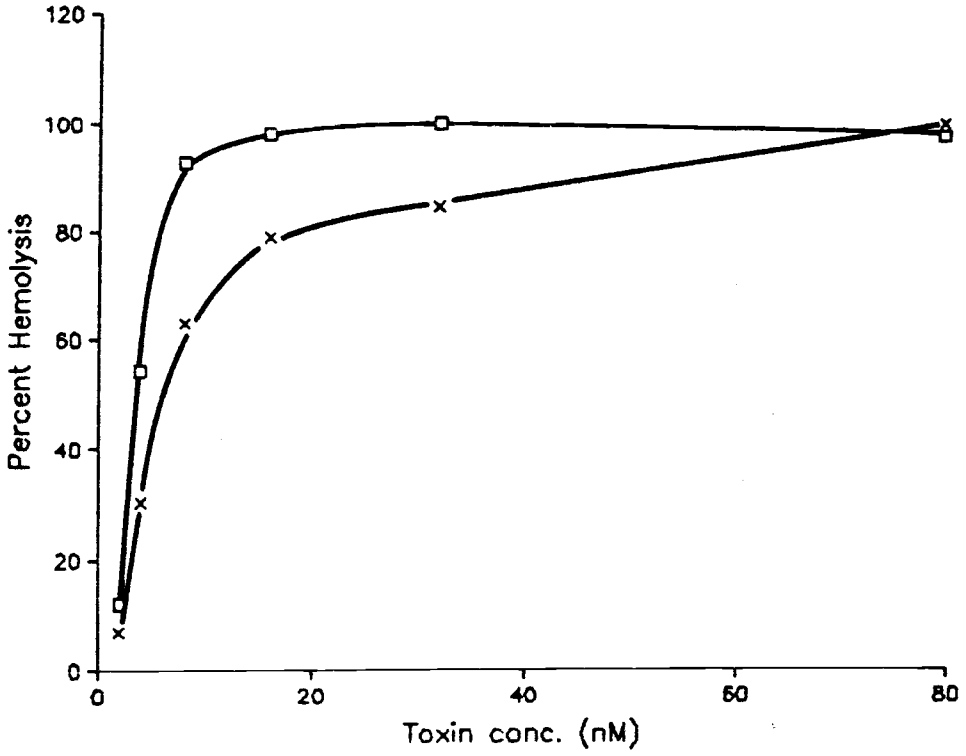

Figure 19. Bti toxin hemolysis of chicken erythrocytes (X) as compared to human erythrocytes (□).

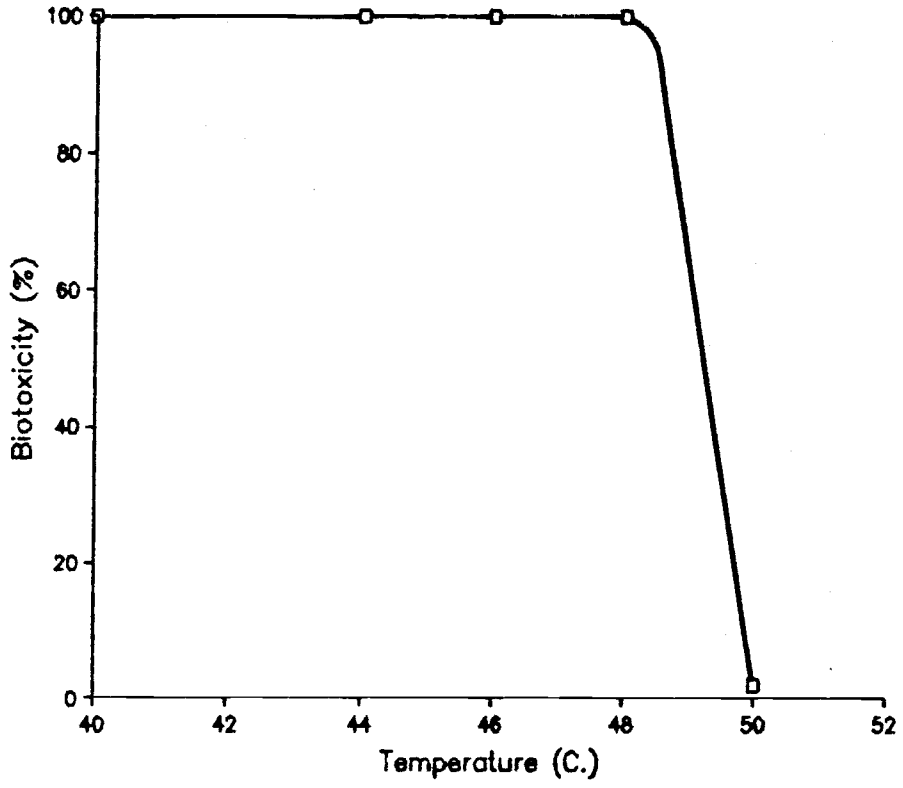

Figure 20. Effect of temperature on Bti toxin *Aedes albopictus* insect cell interaction.

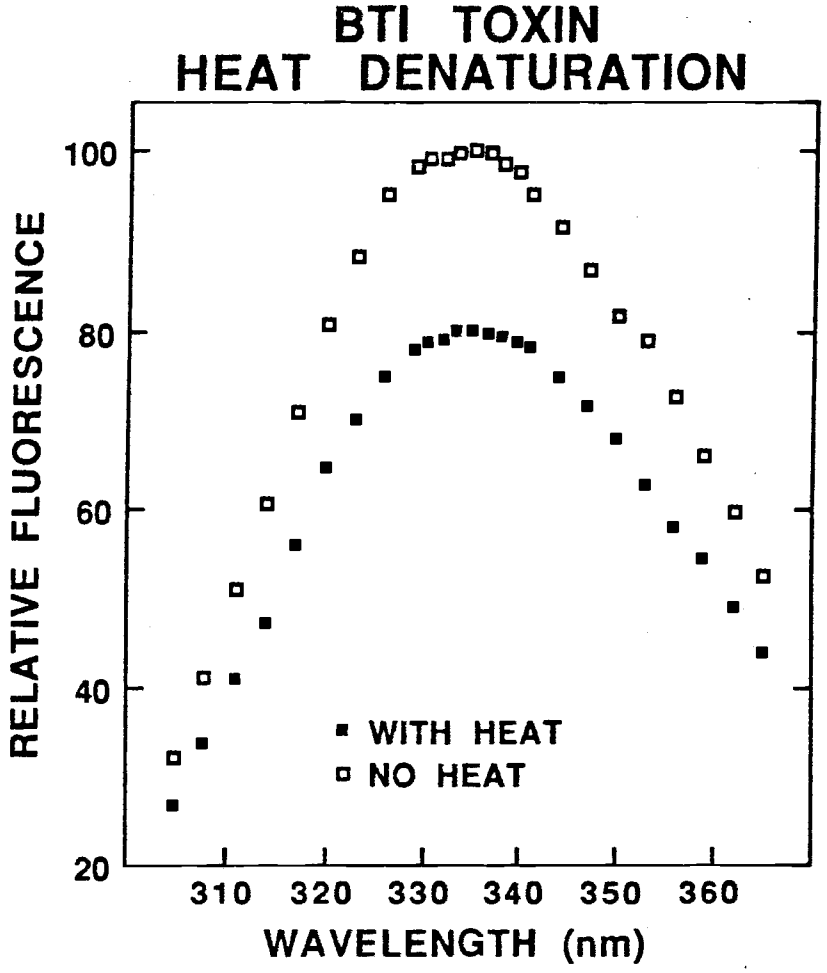

Figure 21. Effect of heat (50°C X 50 minutes) on on Bti toxin denaturation as measured by fluorescence.

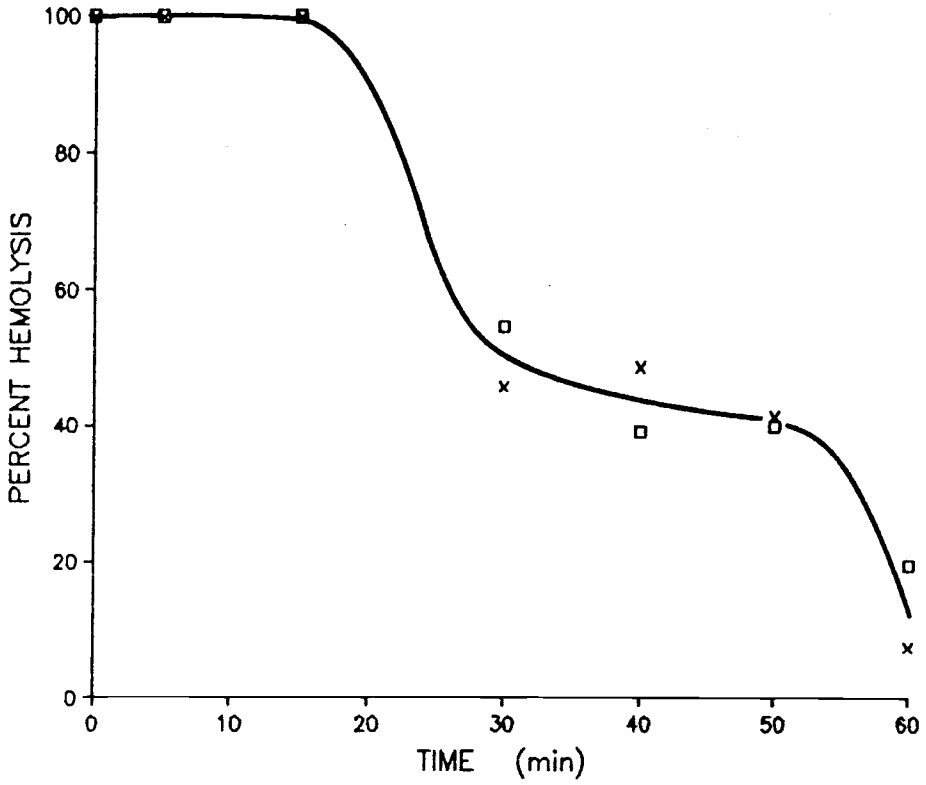

Figure 22. Effect of heat (50°C) on Bti toxin hemolysis.

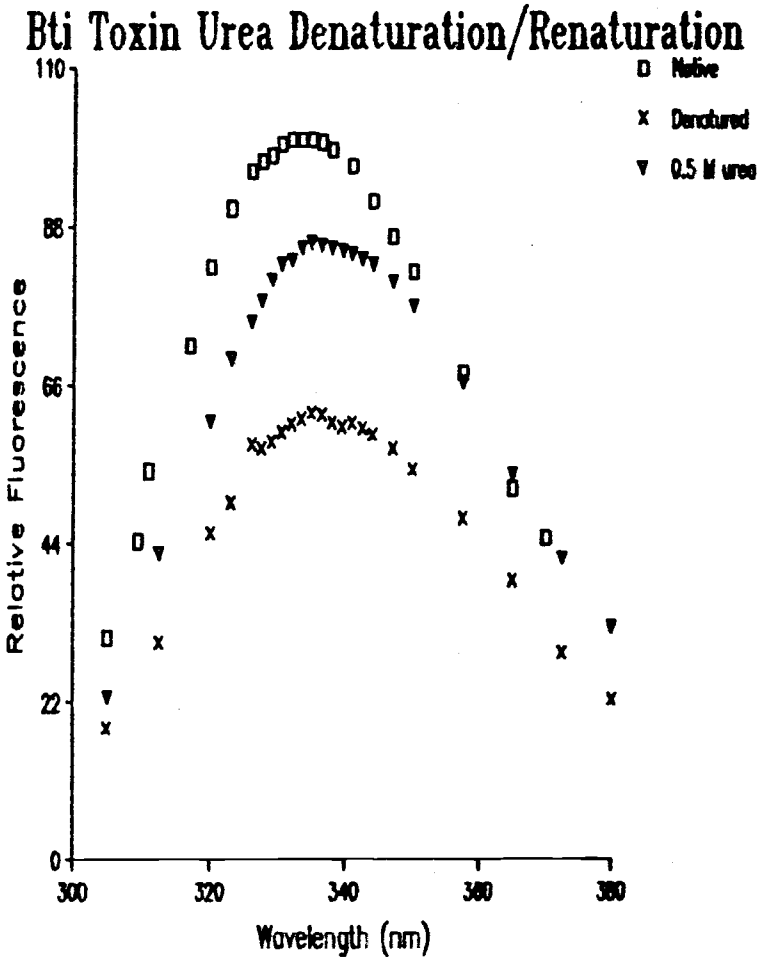

Figure 23. Effect of urea on Bti toxin denaturation and renaturation as measured by fluorescence.

DISCUSSION

The hemolytic bacterial toxins *C. perfringens* alpha toxin and *Staph aureus* beta toxin have been shown to lyse erythrocytes by phospholipase C enzymatic activity (Freer and Arbuthnott 1976, Waisman and Caird 1967). Both of these toxins have demonstrated toxicity to a wide range of eukaryotic cells and are lethal to animals upon in vivo injection (Smith 1975). Under the hemolytic concentrations tested, Bti toxin did not demonstrate phospholipase C activity. Some phospholipase C's require a divalent cation (magnesium or calcium) for optimal activity (Avigad 1976). 10 mM calcium has been found to be optimal for *C. perfringens* phospholipase C (Takahashi et al. 1981). If the Bti toxin did have phospholipase C activity, it was one that did not require divalent metals for activity, because calcium and other metals inhibited hemolytic activity, and EDTA and EGTA did not affect hemolysis. Although an egg yolk contains about 5 mM calcium (U.S.D.A. 1976), it is not likely that this concentration would have inhibited the Bti toxin added at 40 times (160 nM) the amount required to lyse erythrocytes.

Bti toxin could have had a specificity for a particular phospholipid in extremely low concentration in the egg yolk, and not enough turbidity was produced to be detected. *C. perfringens* alpha toxin has substrate specificity for phosphatidyl choline, phosphatidyl serine and sphingomyelin (Stahl 1973). Thus, Bti toxin probably did not have such substrate specificity in the egg yolk nor in the human erythrocyte membrane which contains 33% phosphatidyl choline, 26% sphingomyelin, 14% phosphatidyl serine and 27% phosphatidyl ethanolamine (Colley et al. 1973). The negative results of the egg yolk turbidimetry assay can be considered preliminary evidence that the Bti toxin was not a phospholipase C. More definitive proof of this could be done by analyzing for the breakdown products of phosphorylcholine and 1,2-diglycerides in the erythrocyte membrane

(Colley et al. 1973).

Bti toxin is not likely to be a phospholipase A_2, as this activity has mainly been seen in animal toxins (snake venom and bees) rather than in bacterial toxins (Wells and Hanahan 1969, Shipolini 1971). Extracellular phospholipase D has been found in spider venom and *Corynebacteria*, but no hemolytic activity has been shown (Bernheimer et al. 1985). It is possible that the Bti toxin induced a phospholipase in the erythrocyte membrane. A direct assay of phospholipase would not detect this. *Staph* delta toxin, which is hemolytic (Freer and Arbuthnott 1983), has been shown to stimulate endogenous phospholipase A_2 activity in mouse fibroblasts (Durkin and Shier 1981).

Armstrong et al. (1985), in purifying Bti toxin, used proteinase K and trypsin to digest the crystal, and retained biological activity. It is interesting that although the toxin contained cleavage sites susceptible to proteinase K and trypsin, it was tolerant to these enzymes. This may permit the toxin to survive in the protease-rich insect gut (Armstrong et al. 1985). It was surprising that full renaturation of the toxin molecule was not seen in the urea and heat treatment. The protease resistant toxin was quite thermolabile, with what appeared to be a cooperative denaturation taking place between 48° and 50°C. NaCl in the 100 to 500 mM range did block inactivation at 50°C, but not at a temperature greater than 60°C. Thus, high ionic strength was not able to stabilize the toxin structure after heat treatment.

The heat inactivation of hemolytic activity was biphasic from 30 to 50 minutes, indicating possible formation of a meta-stable toxin that would more likely be able to renature than a totally inactivated molecule. When the partially denatured toxin was examined for renaturation, none was found, and the toxin molecule further lost hemolytic activity in the 4 hour period after removal

of the heat. Thus, even mild denaturation was irreversible and the tendency was for the toxin to be driven toward a totally denatured state.

The excess binding of Coomassie blue dye by the heat and urea-denatured toxin along with the lack of migration into a non-denaturing polyacrylamide gel implied the formation of an aggregated structure as opposed to a monomeric molecule with a reduced negative charge. This aggregated complex was able to be disassociated with SDS treatment as normal migration was seen in a denaturing gel for the urea denatured toxin. This aggregated toxin may have been held together by hydrophobic bonds caused by non-polar amino acids coming to the surface of the molecule.

The Lowry protein assay showed that the same amount of protein was present in the heat-denatured toxin as compared to the native toxin. This meant that the changes in the fluorescence parameters were indicative of a true structural change and that the loss of biological activity was not due to a loss of toxin from solution e.g. adhering to the test tube and cuvette wall. The denatured fluorescence spectra was similar in both the urea and heat treatment. There appeared to be some reorganization of the urea-treated toxin after urea removal as the emission intensity increased about 40%. However, this renatured molecule was not biologically active, therefore correct refolding did not take place.

Pfannensteil et al. (1985) treated Bti toxin with 4, 6 and 8 M urea for 30 minutes. Bioassays of *A. aegyptus* larvae showed 87, 50 and 16% biologic activity, respectively, remaining. This thesis has shown that treatment with 6 M urea for a longer period of time of 15 hours led to a total loss of biologic activity. Pfannensteil et al. (1985) also demonstrated that the Bti toxin was extremely sensitive to 4 M guanidine after a 30 minute treatment as only 10% biologic activity remained. No renaturation studies with urea or guanadine

were attempted by Pfannensteil et al. (1985).

Gasparov et al. (1985) heat-treated the lepidopteran-specific Bt *alesti* toxin, and through circular dichroism measurements, determined that the secondary structure stayed constant to 80°C, but that after 50°C, the tertiary structure of the toxin started to irreversibly change as evidenced by changes in ellipticity at the aromatic absorption wavelength (287 nm). Heating the toxin to 90 to 100°C, caused irreversible tertiary structural changes to occur. The toxin was monitored up to 18 hours after removal of the heat and no renaturation was seen. Bti toxin, which has shown to be protease-resistant (Armstrong et al. 1985), may have a thermodynamically stable and unusual structure in which hydrophilic amino acids such as lysine and arginine which are normally on the surface (Creighton 1983) are hidden and unavailable for interaction with the proteases. If such a structure exists, it was still susceptible to permanent loss of structure and biological activity under the denaturing and renaturing conditions studied. It is not known if the protease resistance of the toxin is necessary for preservation of the toxin molecule inside the parasporal crystal or the insect gut, or if the protease resistance confers a unique structure that is necessary for the toxin-cell interaction. In order to provide more information in this area, future studies could examine the denatured toxin for susceptibility to proteases.

The pH studies showed that the Bti toxin lost hemolytic activity at a pH greater than 9.5 and less than 6. The drop off between pH 9.5 and 10 appeared to be cooperative, similar to that seen in the heat inactivation of insect cell activity from 48 to 50°C, and in both cases, denaturation of the protein appeared to be taking place. The cells did not become irreversibly unresponsive to toxin lysis by pH 10 treatment. The hemolytic activity of the Bti toxin seen at pH 9.5, but absent at pH 10 could have been due to a reversible alteration of the cells. However, the data showing that toxin

treated at pH 10 was inactivated when added to cells that were never exposed to pH 10 strongly suggests that the pH 10 loss of activity was due to an effect on the toxin and not the cells.

The loss of toxin activity at a pH greater than 9.5 was not surprising, considering that the Bti toxin is active in the alkaline dipteran gut (Bulla et al. 1980). This inactivation could indicate a particular requirement for tyrosines (pKa_3 10.07) or lysines (pKa_3 10.53) for the maintenance of a stable protein structure or for a properly functioning active site. The broad pH spectrum of hemolytic activity of the Bti toxin was different from the erythrocyte membrane damaging *Staph* alpha toxin which showed optimum hemolysis of human erythrocytes at pH 5 and a sharp reduction after pH 6 (Bhakdi et al. 1984). The pH reduction seen in inflammatory processes could allow *Staph* alpha toxin to exert its cytotoxic effect at minimal concentrations (Bhakdi et al. 1984). Streptolysin O showed a decrease in hemolytic activity below 6.5, and from 6.5 to 7.5 a plateau was seen. No studies were carried out above pH 7.5 (Oberly and Duncan 1971).

The renaturation of Bti toxin after pH 10 treatment was more successful than with urea or heat. Initially 73% recovery of the biological activity was seen. Hemolytic activity was rapidly lost over the first 10 minutes of renaturation, but from 10 to 60 minutes, the toxin appeared to be in a stable renatured form with 38% activity. Most of the toxin molecule may have initially refolded to a near fully active structure, but there could have still been a small part of the molecule that was not folded correctly, thus causing an unraveling. Similar to the heat renaturation experiment, once the native toxin structure was disrupted, it was not able to renature in a fully active form.

The results of the pH 10 denaturation-renaturation were encouraging since the biological activity went from 4% to 73% upon a change in

pH from 10 to 9. The pH 10 renatured toxin could be studied for longer periods of time to determine if it truly was stable. Allowing renaturation to occur at pH 8 or 7.5 may produce a more biologically active toxin. In addition, renaturation studies using only a partially denatured toxin by exposure to pH 10 for less than 60 minutes, should be undertaken, in the hope of observing more complete reactivation. The sharp drop off in biological activity from pH 9.5 to 10 and the promising data with regard to renaturation provide an excellent system for elucidating properties of the structure of the Bti toxin molecule as well as information which could lead to the determination of domains necessary for biological activity. Gasparov et al. (1985) studied the Bt *alesti* toxin at pH 9 to 12 and found both secondary and tertiary structures changing between pH 10.5 and 11.5. This shift was a little higher than the pH denaturation of Bti toxin as determined by the hemolytic bioassay. Gasparov et al. (1985) did not measure biological activity which could have been lost at a lower pH than the spectroscopic changes.

Toxin concentrations of 4 to 8 nM routinely produced between 80 and 100% hemolysis in 60 minutes. The 4 nM concentration corresponded to a toxin-to-cell ratio of 96,320. 0.44 nM toxin concentration was the threshold level for the detection of lysis in 60 minutes and this corresponded to a toxin to cell ratio of 10,595. Bhakdi et al. (1985) conducted similar toxin-concentration hemolysis studies with *Staph* alpha toxin and found that 8,000 toxin molecules were required to lyse a cell in 90 minutes. This ratio was much lower for Streptolysin O, as only 100 molecules per cell were required to lyse an erythrocyte in 60 minutes, which could be due to the more specific nature of the cholesterol-toxin interaction (Bhakdi et al. 1984). Although 60 minutes was chosen for the Bti toxin hemolysis studies, in 24 hours at 37°C, 0.44 nM toxin produced a full lysis, but 0.04 nM did not. Studies could not be performed past 24 hours because the toxin lost activity from prolonged incubation. Thus, at

least 10,595 molecules per cell were required for lysis in 24 hours. Comparative data was not published for *Staph* alpha toxin or Streptolysin O (Bhakdi et al. 1984, 1985).

Cell concentration was varied over an 18.4 fold range from a toxin-to-cell ratio of 192,640 to 10,470 in an effort to see if the hemolytic phenomenon was saturable, which it was not, as every additional cell added was lysed. It was not likely that the toxin was recycling because the lysate inactivated the toxin. It could be that at 4 nM toxin concentration, the number of toxin molecules present were saturating the number of available sites on the erythrocytes over the entire 18.4-fold range.

When the toxin-to-cell ratio was made 10,595 by lowering the concentration of the toxin, virtually no lysis was seen, yet a similar ratio produced by raising the concentration of cells produced hemolysis indistinguishable from the standard 4 nM assay. At a toxin-to-cell ratio in the range of 10,470 to 10,595, the reaction can be driven by the number of cells or the number of toxin molecules. Thus in one hour, it was crucial to have 4 nM toxin concentration, irrespective of the cell concentration, for a full lysis to occur. Sandler et al. (1985) found Bti toxin hemolysis to be independent of erythrocyte concentration over a 100-fold range. Studies with *Staph* alpha toxin have also shown that the extent of hemolysis was independent of erythrocyte concentration varied over a 3-fold range (Cooper et al. 1964, Jackson and Little 1957). Streptolysin O differs from *Staph* alpha and Bti toxin in that a decrease in the extent of hemolysis with increasing erythrocyte concentration was observed, which could have been due to the specific nature of the interaction of Streptolysin O with membrane cholesterol (Kanbayashi et al. 1972).

The reduction of Bti toxin hemolysis with decreasing temperature has been confirmed by Sandler et al. (1985) who saw a 75% reduction in

hemolysis at 23°C as compared to 37°C. Thomas and Ellar (1983a) could not detect any Bti toxin hemolysis at 4°C; this study similarly showed an absence of hemolysis at 0°C. The loss of activity at 0°C could have been due to the membrane becoming less fluid such that the toxin-membrane interaction was unable to result in lysis. Cooper et al. (1964a) studied the effect of temperature on *Staph* alpha toxin hemolysis from 25 to 52°C and found an increasing lag time and lower extent of hemolysis, as the temperature was decreased, which agreed with the results of this study. When the temperature was increased over 48°C, the hemolysis was reduced and the lag time again increased. Raising the temperature from 37 to 48°C did not increase the extent of *Staph* alpha toxin hemolysis (Cooper et al. 1964a). Bti toxin also appeared to have its maximum hemolytic activity at 37°C. Oberley and Duncan (1971) observed no hemolysis at 4°C with Streptolysin O. Joulet-Reynaud and Alouf (1983) showed binding of the hemolytic ^{125}I *C. perfringens* delta toxin to erythrocytes even at 0°C, concluding that the decreased temperature inhibited an event occurring after binding.

Prelytic leakage of potassium from erythrocytes exposed to a cytolytic agent is indicative of a specific membrane alteration in which a small hole is first created, allowing potassium efflux. This then causes disruption of the osmotic balance of the cell which causes the hole to be enlarged, finally allowing hemoglobin to egress (Weed and Reed, 1966). The similar-appearing efflux curves of potassium and hemoglobin for the Bti toxin indicated that potassium release and hemoglobin release were closely coupled. This indicated that if a small lesion was initially forming, it did not remain small for any length of time and rapidly developed into a larger one, causing cell lysis.

Prelytic potassium leakage has been seen with channel forming *Staph* alpha toxin hemolysis where 50 to 75% of the potassium escaped

before hemoglobin was detected (Madoff et al. 1964). The potassium and hemoglobin release curves for Staph alpha toxin (Madoff et al. 1964) were much more separated in time than the Bti toxin curves reported in this study. Similar to Bti toxin, Jovilet-Reynaud and Alouf (1983, 1984) did not see a difference in the kinetic curves for $^{86}Rb^{+}$ and hemoglobin release for the hemolytic *C. perfringens* delta toxin. The first-order kinetic plot for the Bti toxin showed potassium initially being released at twice the rate of hemoglobin, but this could have been due to the difference in sizes of the two molecules, as the diffusion coefficient is inversely related to the molecular weight (van Holde 1985).

Pfannensteil et al. (1985) incubated Bti toxin with DTT (500 mM) and 2-mercaptoethanol (250 mM) and found no effect on larvicidal activity. This was confirmed in the hemolytic assay with DTT in this study. This was also not surprising, because amino acid analysis of the Bti toxin showed no cysteines (Armstrong et al. 1985). This implied if the Bti toxin was interacting with a specific receptor on the erythrocyte membrane, an intact disulfide bond was not required in the toxin or the membrane. Upon standing, Streptolysin O lost hemolytic activity, which was regained by addition of thiol compounds such as DTT, which implied that activity was dependent upon the cleavage of at least one disulfide bond (Bernheimer 1974, Alouf 1980). Oberly and Duncan (1971) incubated erythrocytes with the thiol-specific reagent N-ethyl maleimide and found no inhibition of Streptolysin O hemolysis.

Bti toxin did not require a non-covalently bound divalent metal as both EDTA and EGTA had no effect on hemolysis. This agreed with the inhibition of lysis that was seen with such divalent metals as calcium, zinc and manganese. The data for magnesium as a weak inhibitor (250 mM gave 59% inhibition) of Bti toxin hemolysis expanded the metal inhibition series proposed by J. Armstrong (unpublished data): La^{+++} --> Zn^{++} --> Mn^{++} --> Ca^{++} --> Mg^{++}.

Bashford et al. (1986) has found that divalent metals inhibited *Staph* alpha toxin hemolysis with an order of potency of $Zn^{++} > Ca^{++} > Mg^{++}$. Hagiwara and Takahashi (1967) have also found magnesium to be the weakest metal inhibitor of the calcium channel of the barnacle muscle fiber, as their rank order potency was $La^{++} \rightarrow Ca^{++} \rightarrow Mn^{++} \rightarrow Ni^{++} \rightarrow Mg^{++}$. However, it is not known if there are any calcium channels in erythrocytes, so the implication of Bti toxin interacting with a calcium channel is strictly speculative at this time.

Although magnesium was a weak inhibitor of Bti toxin hemolysis, it has been shown to be a potent stimulator of lysis as only 8 mM was needed to bring about lysis in a tris-sucrose buffer (J. Armstrong unpublished data). This has also been confirmed with *Staph* alpha toxin by Bashford et al. (1986). Oberly and Duncan (1971) showed that sodium and potassium stimulated Streptolysin O lysis but found magnesium to be equally potent with calcium (25 mM) in inhibiting hemolysis, suggesting that the mode of action of magnesium inhibition with Streptolysin O is different than with Bti toxin.

Cooper et al. (1964b) were able to inhibit *Staph* alpha toxin hemolysis by the presence of 0.3 M sucrose. Bhakdi et al. (1984) saw blockage of *Staph* alpha toxin hemolysis with 30 mM dextran 4 (MW 4000). Molecules such as sucrose, albumin (effective diffusion radius 4.4 and 35.5 angstroms, respectively), and dextrans can rebalance the osmotic loss created by the initial membrane lesion. The size of the molecule that stops hemolysis can be used as an estimate of the lesion size (Seals et al. 1964). The effective diffusion radius of hemoglobin is 32.5 angstroms, and molecules of this size or larger can stop hemoglobin release (but not cation release) (Weed and Reed 1966). The fact that sucrose and albumin did not inhibit Bti toxin hemolysis was evidence that the cell destruction was not proceeding by the colloid osmotic mechanism. This was also in agreement with the closely coupled kinetics of

potassium and hemoglobin efflux implying that a stable channel was not forming.

The lack of inhibition of the purified monoclonal antibody on Bti toxin hemolysis was in agreement with the data of Armstrong et al. (1985), where it was demonstrated that the unpurified monoclonal antibody was non-neutralizing. Whatever site the MAB was binding to was different from the site required for toxic activity. Harshman et al. (1986) found no inhibition of hemolysis when *Staph* alpha toxin was incubated with its monoclonal antibody. Further studies showed that the MAB bound a carboxy fragment of the *Staph* alpha toxin indicating that this part of the molecule was free in solution and was not necessary for receptor binding.

The lack of inhibition of Bti toxin hemolysis by 25 mM ATP may indicate that positively charged sites on the toxin or the erythrocyte membrane were not necessary for hemolysis at pH 7.4. The neuraminidase treatment of the cells did not cause any reduction in hemolytic activity, thus the Bti toxin did not interact with an erythrocyte membrane-receptor containing a terminal sialic acid. It was still possible that the Bti toxin could have interacted with a non-sialic carbohydrate on the erythrocyte membrane.

Jovilet-Reynaud and Alouf (1983) saw a 30% decrease in binding of hemolytic *C. perfringens* delta toxin to neuraminidase-treated sheep erythrocytes. Also, preincubation of the toxin with G_{M2} ganglioside prevented binding and lysis. It was suggested that the *C. perfringens* delta toxin was interacting with a membrane component containing a glycolipid (Jovilet-Reynaud and Alouf 1983). Thomas and Ellar (1983) could not demonstrate any inhibition of cytolysis against insect cells when Bti toxin was preincubated with cerebroside-cholesterol-dicetyl phosphate liposomes. Knowles et al. (1984) found a loss of activity of the Bt *kurstaki* toxin towards *C. fumiferana* cells after incubation with N-acetyl galactosamine, N-

acetyl neuraminic acid, and soybean and wheat germ egg agglutinin. As N-acetylneuraminic acid is not known to occur in insects, this suggested that the specific plasma membrane receptor for the Bt *kurstaki* toxin had a terminal N-acetyl glucosamine residue.

The data on the sensitivity of chicken erythrocytes to Bti toxin expands Thomas and Ellar's (1983) work on rat, mouse, sheep, and horse erythrocytes. Relative sensitivity was not reported in their study. The cells used in Thomas and Ellar's (1983) study were all non-nucleated and this is the first report of the sensitivity of a nucleated erythrocyte to Bti toxin. The extent of hemolysis was less for chicken cells than human cells in the lower toxin concentrations of 40 nM and less. This could have been due to the different lipid receptor make-up of the chicken erythrocyte membrane, or upon hemolysis, Bti toxin may have interacted with protein and nucleic acids from the chicken erythrocyte nucleus which were not present in the non-nucleated human erythrocytes (Ponder, 1948).

The Bti toxin was not binding the erythrocyte membrane tightly, as removal of free toxin in solution after the lag period caused a large reduction in hemolysis. This was different from what has been observed with other bacterial hemolytic toxins. Harshman and Sugg (1985) saw no inhibition of lysis with the same experiment with *Staph* alpha toxin on rabbit erythrocytes, indicating that a toxin-cell interaction was taking place early which was irreversible. Oberly and Duncan (1971) incubated Streptolysin O with erythrocytes at 4°C and no lysis took place, then the cells were resuspended with fresh buffer, the temperature raised to 37°C and a full lysis was observed. It was concluded that there were at least two steps involved in hemolysis: the first being an irreversible adsorption of toxin to the cell, and the second being the membrane damaging step. Jolivet-Reynaud and Alouf (1983) have shown with ^{125}I-labelled toxin, that the binding step for *C. perfringens* delta toxin was

separated temporally from the release of hemoglobin and potassium.

Like Bti toxin, Harshman and Sugg (1985) have also shown that calcium inhibited *Staph* alpha toxin hemolysis in the range of 10 to 50 mM. The calcium inhibition studies for Bti toxin showed that calcium was able to block Bti toxin hemolysis at any time during the kinetic hemolytic bioassay. This was not so with *Staph* alpha toxin. Harshman and Sugg (1985) demonstrated with *Staph* alpha toxin that after the initial binding (lag) period had elapsed, the lysis process was committed and later calcium addition did not have any effect on hemolysis. Harshman and Sugg (1985) concluded the calcium was blocking a post binding event, such as the activation of the *Staph* alpha toxin-membrane complex, because calcium treatment during the lag phase, followed by removal of the calcium (and unbound toxin) did not inhibit lysis. With Bti toxin, calcium could be inhibiting an initial toxin binding (albeit weak) event, or like *Staph* alpha toxin, the calcium could be inhibiting the activation of a Bti toxin-membrane complex. Whatever the case, the binding and Bti complex activation steps appeared to be occurring throughout the assay as opposed to the confinement of these events to the lag phase, as seen with *Staph* alpha toxin. Calcium (as well as the other divalents such as zinc and manganese) could also have competed for some type of anionic site with which the Bti toxin also interacted. This competition by calcium could have had an effect throughout the assay.

Calcium did not irreversibly affect the Bti toxin or the erythrocytes, but a reversible effect on either could have taken place. Incubation of Bti toxin with calcium did not affect the intrinsic fluorescence of the Bti toxin (M.L. Pollock unpublished). Thus, it was likely that calcium could have still somehow inactivated the toxin membrane complex as has been proposed by Harshman and Sugg (1985).

Staph alpha toxin is known to form hexamers on the erythrocyte membrane which are responsible for channel formation that eventually cause lysis (Fussle et al. 1981). Structures consisting of between 25 and 100 Streptolysin O molecules, thought to be channels, have also been seen on the erythrocyte membrane (Bhakdi et al. 1984). Ellar et al. (1985) have proposed membrane insertion of one or more Bti toxin molecules to form a channel. If polymerization of the Bti toxin was taking place, it is not known if this was occurring in solution or on the membrane. The divalent metal ion inhibition could have been acting on either one of these processes.

Monovalent ions and Mg^{++} stimulated Bti toxin hemolysis and this may have been due to a catalysis of a monomer ---> oligomer reaction. Another possibility is that the monovalents and Mg^{++} could have made the membrane more receptive to the toxin as has been suggested with *Staph* alpha toxin (Bashford et al. 1986). Denaturation data showed a proclivity for Bti toxin to form aggregates but these must not have been the activated form because they were biologically inactive. The loss of hemolytic activity at between pH 6 and 9.5 may have been due to the denatured toxin being unable to form active oligomers needed to cause a membrane lesion. The monomer ---> oligomer transition has also been seen in the cytolytic action of human complement where 12-18 of the 71 Kd monomers form channels in the cell membrane (Podach 1986).

Pfannensteil et al. (1985) modified lysines on the Bti toxin and found loss of larvicidal activity with O-methylisourea (45% activity), potassium cyanate (30%), succinic anhydride (12-64%), and dansyl chloride (6-100%). When Bti toxin lysines were biotinylated in preparation for avidin-biotin fluorescence binding studies, loss of biological activity was seen, confirming Pfannensteil et al.'s observations (1985). There are 10 lysines on the Bti toxin molecule (Armstrong et al. 1985) and it is not known how many of them were essential for activity because the uptake of biotin was not measured

nor was it in the modification carried out by Pfannensteil et al. (1985). However, at least one lysine (or the amino terminus) was essential to produce hemolysis. This lysine modification and the resultant loss of hemolytic activity was also in agreement with the pH studies where there was a loss of activity between 9.5 and 10. This modification needs to be carried out at a lower biotin-to-toxin ratio in order to produce a biotinylated Bti toxin that is biologically active, and thus useful for defining the Bti toxin membrane interaction.

The FITC-toxin binding seen late in the hemolysis assay appeared to be to lysed cells, not intact ones. This suggested a low level of binding of the toxin during the initial 10 minutes, which was implied with the buffer resuspension experiments. McCarthy and Arbuthnott (1978) used FITC anti-horse gamma globulin (indirect method) on human and rabbit erythrocytes and was only able to generate a small amount of binding in the beginning and the end of hemolysis with *Staph* alpha toxin. Also Klainer et al. (1964) in an immunofluorescence study with *Staph* alpha toxin and human erythrocytes was only able to demonstrate binding in the middle portion of the hemolytic process, not at the beginning.

Singh et al. (1985) and Chilcott et al. (1984) reported neural transmission blockage after Bti toxin administration to the cockroach. English and Cantley (1985) have found Bt *kurstaki* toxin inhibited both potassium transport in *Manduca sexta* insect cells and a K^+/H^+ ATPase isolated from these cells. However, Bti toxin did not show any effect on erythrocytes treated with potassium-, sodium-, calcium-, or anion- channel blockers, as well as ouabain, an antagonist of the Na^+/K^+ ATPase. Ozaki et al. (1985) have shown that the marine coelenterate palytoxin lysed erythrocytes at 1 nM concentrations and interacted with the Na^+/K^+ ATPase, as 20 uM ouabain caused a loss of hemolytic activity. The lack of inhibition of Bti toxin hemolysis with ouabain agreed with the data showing

that the response of ATP-depleted and fresh cells to Bti toxin was the same. This suggested that the toxin was not interacting with an erythrocyte membrane ion pump requiring ATP. The similar kinetics of the bioassay using either 150 mM sodium or 150 mM potassium was also evidence that the Bti toxin was not interacting with a pump involving potassium.

The diuretic, furosemide, has been reported to inhibit ion transport activity in the erythrocyte in two ways. The first is by inhibition of the anion channel, through which anions such as chloride and sulfate are transported (Brazy and Gunn 1976). The inhibition of furosemide on the erythrocyte anion channel is similar to DIDS (Narahashi et al. 1964) The second action of furosemide on erythrocyte ion transport is inhibition of $Na^+/K^+/2Cl$ cotransport. This transport pathway, involving the movement in tandem of a sodium, potassium and two chloride ions, is used as a way for the erythrocyte to regulate its volume (Wiley and Cooper 1974). DIDS did not show any inhibition of Bti toxin hemolysis. This suggested that if the 66% Bti toxin hemolysis seen in the presence of 7.5 mM furosemide was indicative of a specific ion transport interaction, the toxin may have been affecting the sodium-potassium cotransport. However, the high concentration of furosemide required to produce a substantial inhibition of Bti toxin hemolysis was 7.5 times the 90% inhibition concentration reported in the literature. Furosemide was probably inactivating the toxin or the erythrocyte membrane in a non-specific manner, making it unlikley that Bti toxin was interacting specifically with the erythrocyte sodium-potassium cotransport pathway.

Considering the lack of evidence for the Bti toxin in causing erythrocyte lysis by interaction with an ion channel or pump, the neural blockade in the cockroach seen by Singh and Gill (1985) and Chilcott et al. (1984) may have been due to a direct destruction of the nerve cells themselves, as the Bti toxin is known to be toxic to

a wide range of mammalian cells (Thomas and Ellar, 1983a) or the toxin's mechanism of disruption on nerve cell conduction was different from that of erythrocytes.

Table 19 lists what is known of the Bti toxin with regard to hemolysis and some properties of the protein, and also compares what is known of 3 other hemolytic bacterial toxins: *Staph* alpha toxin, *C. perfringens* alpha toxin and Streptolysin O toxin. Figure 24 is a proposed model, which summarizes the author's view of the interaction of the Bti toxin and the erythrocyte based on the data of Table 19. Figure 24-1 represents the large numbers of toxin molecules necessary to be in solution in order to observe lysis. The high toxin-to-cell ratio could be required to drive the formation of an oligomeric intermediate, shown as a trimer (but could be made up of any number of toxin molecules) in Figure 24-2. The purpose of the oligomer is to create a toxin structure that would be better able to insert, damage the membrane, and create a lesion than individual monomers. The denaturation of the protein seen at pH 10 could make oligomer formation impossible. Figure 24-2 also shows the monoclonal antibody (MAB) binding site which must be different from the membrane interaction site, as the MAB was non neutralizing.

The activated oligomeric Bti toxin complexes are indicated by the triangles in Figure 24-3 and they interact with the erythrocyte membrane as shown in Figure 24-4. Figure 24-2 shows the activated toxin oligomer forming in solution but it could also be forming directly on the membrane. Calcium, by interacting with the cell membrane, could stop this interaction from occurring, or the calcium could stop the progression of a toxin-erythrocyte complex already formed from completing its action (Figure 24-4). It is the large amounts of toxin molecules in solution (Figure 24-1) that continually drive the toxin-cell interaction and addition of calcium in Figure 24-4 will stop the process at any time. Sodium and

potassium, as well as magnesium could somehow stimulate the lytic process by making the membrane receptive to the Bti toxin. Lowering the temperature decreases the kinetics of lysis because the membrane becomes more rigid which will hinder the toxin-membrane complex from forming, or once formed, will prevent the complex from lysing the cell (Figure 24-5).

The interaction of the Bti toxin with the erythrocyte is weak and transitory. The toxin hits the cell and is quickly released and leaves a small lesion (Figure 24-5). The process then repeats itself (Figure 24-6 to 24-7). Finally after a number of membrane punctures, the erythrocyte starts to become leaky and potassium efflux begins (Figure 24-8). This is followed rapidly by hemoglobin release which brings about cell destruction. FITC-toxin binding was not detected in the early part of the hemolysis and appears to be binding to lysed membranes as shown in Figure 24-10.

TABLE 19

Comparison of Bti toxin with *Staph* alpha, *C. perfringens* alpha and Streptolysin O toxin

I Hemolysis	Staph alpha	C. Perfrin. alpha	Streptolysin O
a. Characterization			
Toxin/cell ratio for 100% lysis in 60 min. (96000)	8000		100
pH inactivation < 6, > 9.5	>6		<7
pH 10 inactivation somewhat reversible			
Lysis independent of cell concentration over 18.4 fold range	yes		no
Lysis decreases below 37° with no change from 37° to 47°C	yes		
Prelytic K^+ release	yes		
Toxin removal decreases lysis	no		
No early fluorescence binding	yes		
b. Substance/Treatment Studies			
40mM Ca^{++} inhibits lysis at beginning	yes		yes
40mM Ca^{++} inhibits lysis at any time	no		
$Zn^{++} > Ca^{++} > Mg^{++}$ inhibit lysis	yes		
Na^+, K^+, Mg^{++} stimulate lysis	yes		no[1]
MAB non neutralizing	yes		
No requirement for -S-S- divalent metals or + charges	yes	no	yes
No colloid osmotic effect	no		
Lysate inhibits	yes		no
No sialic receptor			
No ion channel (Na^+, K^+, Ca^{++}, or anion)			
No ion pump (Na^+/K^+ ATPase)			
Possibly Na^+/K^+ cotransport			
No specificity for Na^+ or K^+			
Chicken erythrocytes less sensitive	no		
II Properties of Bti Toxin			
6 M urea or heat (50° X 50 min.) Denaturation			
No renaturation from heat or urea			
Tendency to form aggregates			
Lysines essential			
Not phospholipase C		no	
3 tryptophans/toxin molecule			

1 20 mM Mg^{++} inhibits lysis 80%

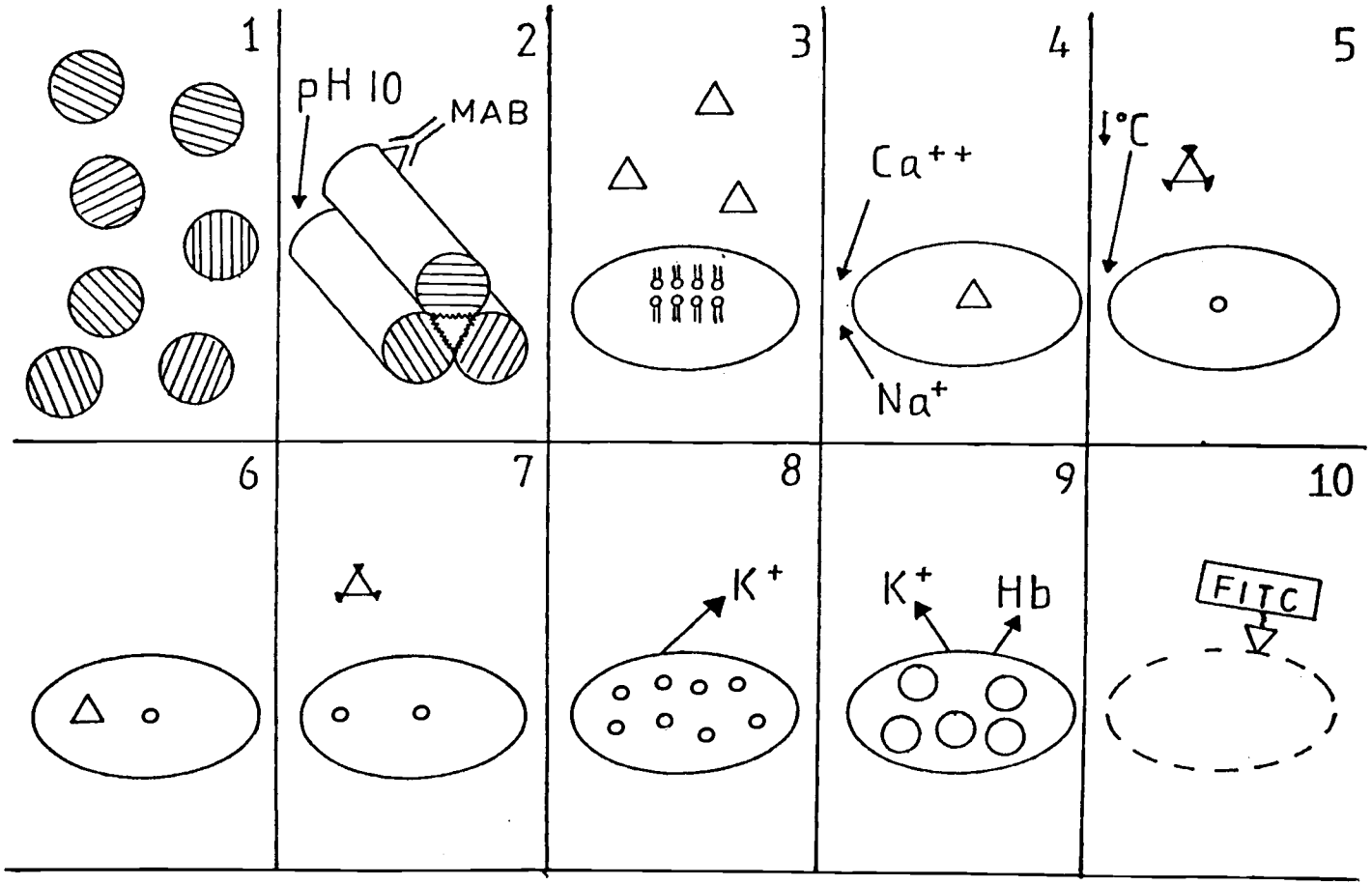

Figure 24. Model for Bti toxin-erythrocyte interaction.

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APPENDICES

APPENDIX A

Aedes Insect Cell Medium

Organic salts medium*	22 ml
10X Medium 99 (lacking glutamine and $NaHCO_3$) (Microbiologic Associates, Bethesda, M.D.)	4.4 ml
0.2M glutamine	1 ml
Fetal bovine serum	10 ml
Penicillin 25,000U/ml and Streptomycin 25,000ug/ml solution	1 ml
Distilled water to make	100 ml

*Organic salts medium

NaCl	16 gm
KCl	0.8 gm
$CaCl_2(H_2O)_2$	0.28 gm
$MgSO_4(H_2O)_7$	0.2 gm
$MgCl_2(H_2O)_6$	0.2 gm
Na_2HPO_4	0.1 gm
K_2HPO_4	0.12 gm
Dextrose	2 gm
Lactalbumin hydrolysate (Difco Labs, Detroit, M.I.)	10 gm
Distilled water to make	1 liter

The solution was adjusted to pH 6.4 and autoclaved for 15 minutes.

APPENDIX B

Nucleotide Sequence of 28 Kd Protein from Waalwijk et al. (1985)

MENLNHCPLEDIKVNP$KTPQSTARVITLRVEDPNEINNLLSI
NEIDNPN*ILQAIMLANAFQNA

LVPTSTDFGDALRFSMPKGLEIANTIRPMGAVVS*VDQNVT2QTNNQVSVMINKLEVLKTVLGVA

LSGSVIDQLTAAVTNTFTNLNTQKNEAIFGKETANQTN*T*NVLFAIQNAQTGGVM*CVPVGF

EIKVSAVKQQVLFFTIQDSAS*NVNIQSLKFAQPLVSSSQ*PIADLTSAINGTL

* = tyrosine

$ = tryptophan

K = lysine

Initial underlined sequence is not needed for toxicity.