Extrapolation of this model to that of a DNA–daunomycin complex shows several potentially interesting features. Since ring D of the drug protrudes out from the helix, substituents attached to C(1) or C(2) [and possibly even C(3)] would not be expected to alter significantly the binding of such drugs to DNA. Specific probes could therefore be attached to these sites without altering the drug–DNA interaction. Attempts to fluorinate daunomycin at these sites are in progress, since the fluorine can be monitored by ^{19}F NMR, which is especially sensitive to the polarity of its immediate environment.

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Binding of *Clostridium perfringens* [^{125}I]Enterotoxin to Rabbit Intestinal Cells†

James L. McDonel

ABSTRACT: ^{125}I-Labeled enterotoxin from *Clostridium perfringens* was utilized to characterize the association of the enterotoxin with cells isolated from rabbit intestine and tissue homogenates from liver, kidney, and brain. The enterotoxin was found to bind in a specific and saturable manner to cells from intestine and to tissue homogenates from liver and kidney but not the brain. Detailed studies of the binding were carried out with the ileal epithelial intestinal cells. The rate and amount of binding of enterotoxin to cells appeared to be temperature dependent. Apparent affinity and association and dissociation rate constants were calculated for what appeared to be two classes of saturable binding sites. The amount of enterotoxin molecules that bound per milligram of cell protein was similar in tissue of intestinal, liver, and kidney origin (approximately 10^{13} molecules/mg of cell protein). Spontaneous dissociation into the supernatant medium was observed to be much slower than expected from calculations based on the rate of association. Chaotropic ions did not enhance dissociation of the enterotoxin from cells. Enterotoxin binding was demonstrated to be heat labile (binding ability was lost after the enterotoxin was heated for 10 min at 60 °C). A mechanism is described whereby the enterotoxin binds and then is inserted into the membrane where it becomes trapped.

Recent studies on the mechanism of action of *Clostridium perfringens* enterotoxin have indicated that it acts through destructive interaction with cellular membranes in susceptible cells (McDonel, 1979). These effects have been noted in rat (McDonel, 1974; McDonel & Duncan, 1975) and rabbit (McDonel & Duncan, 1977; McDonel et al., 1978) intestinal models and in Vero (African green monkey kidney) cells grown in culture (McClane & McDonel, 1979, 1980; McDonel & McClane, 1979). The results of preliminary studies with [^{125}I]enterotoxin in Vero cells (McDonel & McClane, 1979)

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suggest that the enterotoxin binds to cells in a "specific" (binding inhibitable by pretreatment with native enterotoxin) manner and that its capability to induce a biological response in cells depends upon the amount bound.

The purpose of the studies presented in this paper is to demonstrate and characterize the binding of the enterotoxin to rabbit intestinal cells, which are the site of action in *C. perfringens* induced disease.

Materials and Methods

Radiolabeling of Enterotoxin. Highly purified enterotoxin (M_r 35 000) was prepared as described (Stark & Duncan, 1972) and its biological activity determined in erythemal units [EU, an index of erythema induced in guinea pig skin by the enterotoxin (Stark & Duncan, 1971)]. Most data in this report are expressed as a function of EU because the specific activity (EU/mg) of the enterotoxin varies from one preparation to another. It has been demonstrated that biological effects, membrane damage, and binding to cell membranes are directly related to biologically active enterotoxin (as measured by erythemal activity), not necessarily total enterotoxin mass (McClane & McDonel, 1979; McDonel & McClane, 1979; McDonel, 1980). Usually, 2 mg of protein was labeled with 2 mCi of reductant and carrier-free sodium iodide-125 (New England Nuclear, specific activity 17 Ci/mg) by the chloramine-T method. Enterotoxin (2 mg in 500 μL of PBS, pH 7.4; see below) was added to the iodine-125 followed by addition of 100 μL (1 mg/mL) of chloramine-T solution. After the desired reaction time at 24 °C, 100 μL of sodium metabisulfite (5 mg/mL) was added to stop the reaction. The contents of the reaction vial were then immediately transferred to a Sephadex G-25 column that had previously been equilibrated with 2% bovine serum albumin in phosphate-buffered saline (PBS) (NaCl, 154 mM; Na_2HPO_4, 10 mM; pH 7.4). The specific activity of the labeled enterotoxin (2000–3000 Ci/mol) after 5-min exposure to the chloramine-T was relatively low. However, increasing the time of exposure to the oxidant resulted in too slight of an increase in specific activity to justify the increased likelihood of causing changes in the enterotoxin's structure or function. The fractions containing labeled enterotoxin were pooled and concentrated against poly(ethylene glycol) Compound 20 M (Union Carbide Corp.) at 4 °C for 2 h. The labeled enterotoxin was then distributed into polyethylene microcentrifuge tubes and stored at −20 °C until used. All samples were used within 2 weeks of preparation. No changes in biological activity or physical characteristics of the protein were detected within this period of time. The biological activity (specific activity 1600–2500 EU/mg) of the enterotoxin was found not to be altered by the iodination procedure. The labeled enterotoxin was further analyzed by cold trichloroacetic acid precipitation, coprecipitation with native enterotoxin, polyacrylamide disc gel electrophoresis, and microslide immunodiffusion (Stark & Duncan, 1971).

Preparation of Cells for Binding Studies. Rabbit intestinal epithelial cells were collected by a method similar to that described (Levine & Weintraub, 1970) which makes use of low-amplitude, high-frequency vibrations generated by a Vibro Model E1 mixer (Chemapec Inc., Hoboken, NJ 07030). New Zealand white rabbits (2–3 kg) were fasted (with water given ad libitum) for 36 h prior to being anesthetized by intravenous injection of sufficient sodium pentobarbital to suppress the animal's pedal reflex. The ileal section of the small intestine was exposed, cannulated, and washed free of luminal debris with oxygenated Ringer's solution at 37 °C (McDonel, 1974). The anterior mesenteric artery was tied off and cannulated immediately after removal of luminal debris from the intestine to avoid contamination of epithelial cell preparations with red cells which tended to be liberated from the lamina propria during shaking. The artery was then flushed with Ringer's solution until the mesenteric arteries and veins were free of blood. A section of terminal ileum approximately 35 cm long was removed and everted over a copper spiral for shaking. Sections were shaken in fructose (20 mM)–PBS at an amplitude of 2 mm for 20 min at 24 °C. The liberated cells were pelleted and washed 3 times. After being counted with a Coulter counter, they were distributed into 1.5-mL polyethylene microcentrifuge tubes at the desired concentration in fructose–PBS. Cell viability at this point was determined by trypan blue dye exclusion to be 85% (range 75–90%) on the average.

Tissue homogenates from liver, kidney, and brain were collected as follows. The liver and kidneys first were perfused with fructose–PBS to remove red blood cells. Then the liver, kidney, and brain were separately minced with scissors and homogenized with a Waring Blendor. The homogenates were passed through cheesecloth and alternately pelleted and washed until the supernatants were clear (usually five or six washes). The washed pellets were resuspended in fructose–PBS and distributed into microcentrifuge tubes.

Protein was determined in all experiments, after the pellets and bovine serum albumin standards were dissolved in 1 N NaOH, by the method of Lowry et al. (1951).

Binding Assay. The desired amount of the [^{125}I]enterotoxin was mixed with cells suspended in 0.5 mL of fructose–PBS in 1.5-mL polyethylene microcentrifuge tubes. All experimental points were obtained in duplicate. Furthermore, at each experimental point [^{125}I]enterotoxin was added to a third tube of cells that had been pretreated for 60 min with an excess of native enterotoxin. After incubation with shaking at the given temperature, the cells were pelleted (20 s) with a Brinkman Model 3200 microcentrifuge, washed once, and transferred to clean microcentrifuge tubes. After the wash supernatant was removed, the pellets and the supernatants were counted separately in a Packard Model 578 auto-γ scintillation spectrometer. "Specific" binding was calculated by subtracting the counts associated with cells pretreated with native enterotoxin ("nonspecific" binding, usually 1–3% of total counts added) from the counts associated with cells not pretreated with native enterotoxin. Statistical analyses were done by using Student's t test.

Dissociation of [^{125}I]enterotoxin from cells was measured directly by three methods. The first measured spontaneous dissociation in the same system as the binding studies were performed. After cells were labeled for 1 h with an excess of [^{125}I]enterotoxin, they were washed and placed into clean tubes. An excess of native enterotoxin was added to the washed cells to facilitate dissociation. At timed intervals, cells were pelleted followed by separate counting of the pellets and supernates. The second method used was identical with the first except that chaotropic salts (0.5 M LiCl or 0.5 M KCNS) were added to the washed labeled cells. The third method was adapted from the membrane filter technique described by Cuatrecasas & Hollenberg (1976) in which labeled cells were placed on membrane filters and buffer was allowed to percolate past the trapped cells. Filters were removed at timed intervals for counting label that remained associated with the cells.

Enzymatic treatment of cells was accomplished by mixing trypsin (0.4 mg/mL) or Pronase (0.2 mg/mL) with cells for 5 min before addition of [^{125}I]enterotoxin or 5 min after binding was completed (1 h). Protease was inactivated and

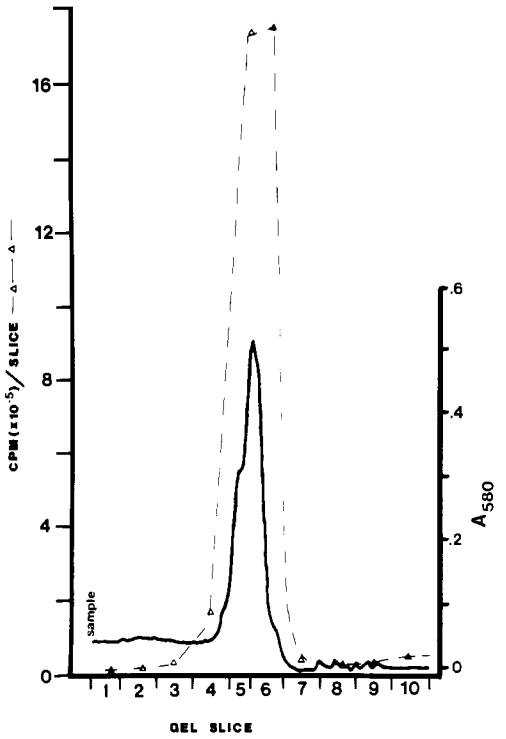

FIGURE 1: Polyacrylamide disc gel electrophoresis of [^{125}I]enterotoxin and distribution of radiolabel. Samples were added to gels at the end marked "sample" (cathode). Gel slices were approximately 2 mm in length each. Electrophoresis was performed as previously described by Stark & Duncan (1971).

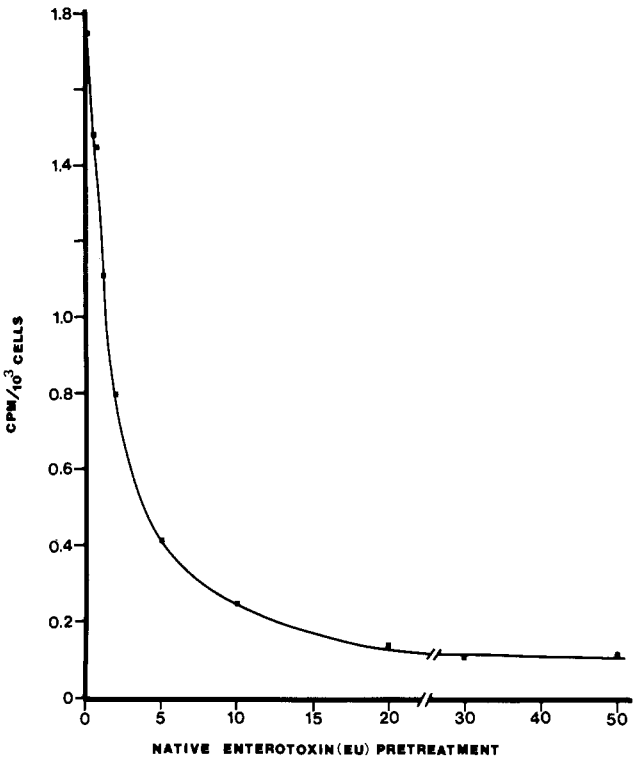

FIGURE 2: Effect of native enterotoxin on the binding of [^{125}I]-enterotoxin to intestinal epithelial cells. Indicated quantities (1 EU = 0.5 μg or 2.9×10^{-11} M in 0.5 mL) of native enterotoxin were added to duplicate 1.5-mL polyethylene microcentrifuge tubes containing 0.5 mL of PBS and cells (2.6×10^7 cells/mL) and incubated at 24 °C with shaking for 60 min followed by addition of 1.0 EU of [^{125}I]enterotoxin (specific activity 1750 cpm/pmol) to each tube. The cells were incubated for another 60 min at 24 °C with shaking. Cells were then pelleted and transferred to clean tubes. Pellets and supernatants were counted separately in a γ counter.

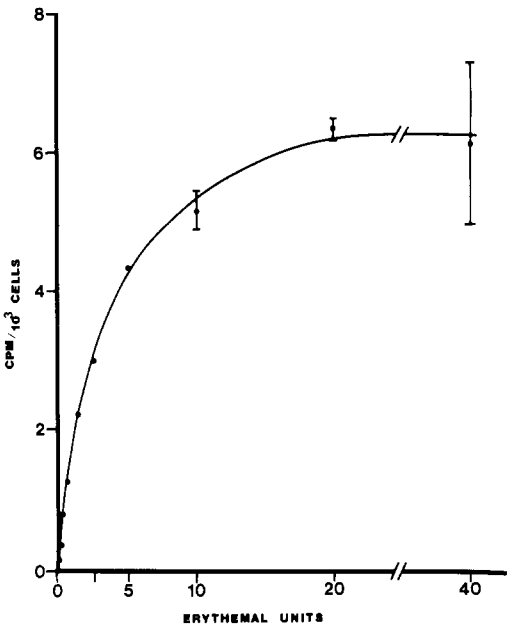

FIGURE 3: Effect of [^{125}I]enterotoxin concentration (1 EU = 0.5 μg or 2.9×10^{-11} M in 0.5 mL) on specific binding to intestinal cells. Duplicate microcentrifuge tubes containing 2.4×10^6 cells in 0.5 mL of PBS were treated with increasing amounts of [^{125}I]enterotoxin (specific activity 1750 cpm/pmol). A third tube for each concentration of labeled enterotoxin had been pretreated for 60 min with 30 EU (15 μg or 8.5×10^{-10} M) of native enterotoxin to correct for nonspecific binding. All tubes were incubated with shaking for 60 min at 24 °C. Counts associated with cells were determined by pelleting the cells, washing them once, and transferring them to clean tubes for separate counting of pellets and supernatants in a γ counter. Error bars show standard error of the mean, which if not shown was smaller than the symbol.

removed by two washings with sucrose–PBS containing 5% calf serum followed by two washings with fructose–PBS.

Results

Analysis of [^{125}I]*Enterotoxin.* Ninety-five percent of the total counts were precipitable with cold 10% trichloroacetic acid or rabbit antienterotoxin serum. Polyacrylamide disc gel electrophoresis (Figure 1) of the [^{125}I]enterotoxin produced a peak indistinguishable from that of native enterotoxin. The slight cathodal shoulder on the peak has been described for enterotoxin in previous studies (Stark & Duncan, 1972). It also can be seen that the radiolabel was found in the gel slices containing the protein. Analysis of the labeled enterotoxin by sodium dodecyl sulfate gel electrophoresis gave similar results (not shown). Through analysis (not shown) by microslide immunodiffusion (Stark & Duncan, 1971) with rabbit antienterotoxin serum, it was found that labeled and native enterotoxin showed a reaction of identity. Both forms lost serological activity when heated at 60 °C for 10 min.

Figure 2 shows that binding of [^{125}I]enterotoxin to intestinal epithelial cells was displaced sharply by pretreatment with increasing amounts of native enterotoxin. The binding that was uninhibitable was taken to be nonspecific. Approximately 90% of the total counts that bound to cells were attributable to specific binding (inhibitable by pretreatment with an excess of native enterotoxin).

The effect of [^{125}I]enterotoxin concentration on the amount of binding is shown in Figure 3. Specific binding increased as a function of [^{125}I]enterotoxin concentration up to 20 EU (12 μg/0.5 mL), at which point saturation of sites appeared to occur. The maximum number of molecules bound was calculated to be 1.59×10^6/cell.

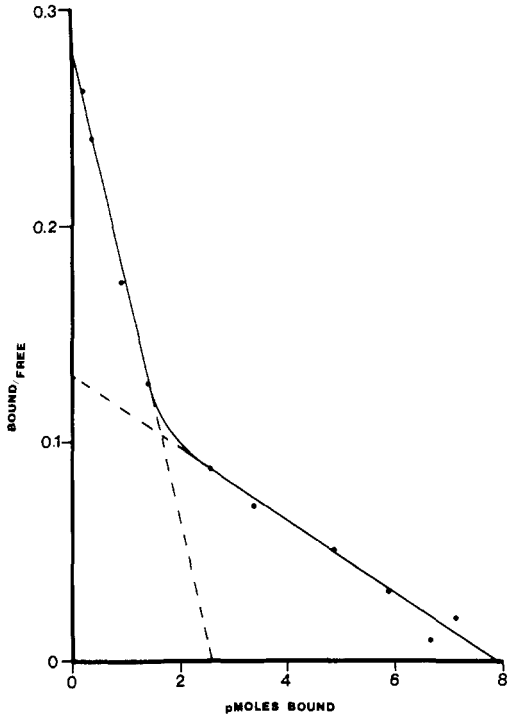

FIGURE 4: Scatchard plot of specific binding data from Figure 3. These data were used to calculate apparent association and dissociation constants (K_a) and binding site densities. The biphasic curve with upward concavity has been construed to imply the existence of two sites with different affinities (high and low).

Table I: Kinetic Constants for [^{125}I]Enterotoxin Interaction with Rabbit Intestinal Epithelial Cells

constant	temp (°C)	high-affinity site	$t_{1/2}$ (min)	low-affinity site	$t_{1/2}$ (min)
apparent affinity constant, K_a	24	5.66×10^7 L/M		8.22×10^6 L/M	
apparent dissociation constant, K_D	24	1.77×10^{-8} M		1.22×10^{-7} M	
association rate constant, k_1	37	2.35×10^5 mol^{-1} s^{-1}	1.1	2.77×10^4 mol^{-1} s^{-1}	20.2
	24	5.94×10^4 mol^{-1} s^{-1}	1.2	7.60×10^3 mol^{-1} s^{-1}	17.0
	4	1.96×10^4 mol^{-1} s^{-1}	1.2	1.41×10^3 mol^{-1} s^{-1}	27.5
dissociation rate constant,[a] k_{-1}	24	1.05×10^{-3} s^{-1}	11.0[b]	9.27×10^{-3} s^{-1}	1.2[b]
sites/cell		0.64×10^6		1.33×10^6	

[a] Dissociation rate constants were calculated from $K_D \times k_1$. These values do not necessarily agree with experimental observations (see text). [b] Calculated from $t_{1/2} = (\ln 2)/k_{-1}$.

A replot of the data in Figure 3 by the method of Scatchard (1949) is shown in Figure 4. The resultant biphasic curve suggests the existence of two classes of binding sites: a high-affinity, low-capacity site with an apparent affinity constant (K_a) (Table I) of 5.66×10^7 L/M and a low-affinity, high-capacity site with an apparent affinity constant of 8.22×10^6 L/M. It can be calculated (Table I) that there are approximately 2 times as many of the low-affinity sites as there are high-affinity sites (a total for both classes of 1.97×10^6 sites/cell).

The effect of cell concentration on [^{125}I]enterotoxin binding to intestinal cells is shown in Figure 5. The increase in counts bound was directly proportional to the increase in cell concentration (expressed as milligrams of cell protein) until an

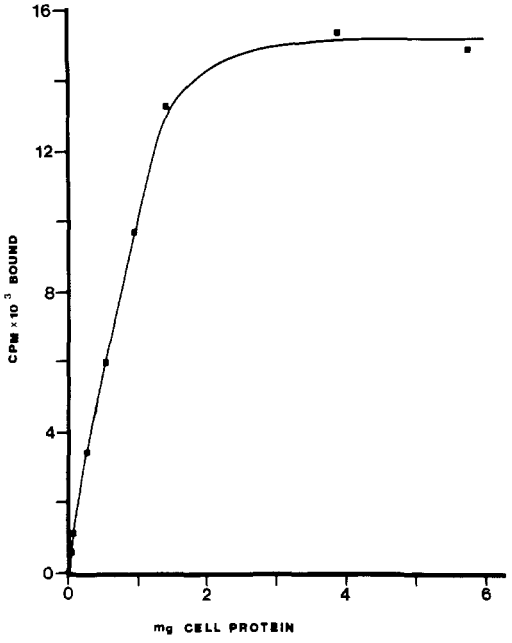

FIGURE 5: Effect of cell protein concentration on specific binding of [^{125}I]enterotoxin. The indicated amounts of cellular material (average number of cells per milligram of cell protein was 8.14×10^6) in 0.5 mL of PBS were incubated with shaking at 24 °C for 60 min in the presence of 1 EU (0.5 μg) of [^{125}I]enterotoxin. Nonspecific binding was determined in the presence of 100 EU (2.9×10^{-9} M) of native enterotoxin for cell suspensions over 2 mg of cell protein/mL and 50 EU (1.4×10^{-9} M) for suspensions under 2 mg of cell protein/mL. Cells (bound label) were separated from unbound label by centrifugation and washing as described in the text.

excess of cellular material was reached. The maximum amount of counts that could be bound from an enterotoxin preparation, even in the presence of excess cellular material, was 69% of the total counts added. This implies that 31% of the counts were associated with enterotoxin that was unable to bind for some reason. This compares to 20% of the counts associated with ^{125}I-labeled cholera enterotoxin being unable to bind to cells (Cuatrecasas, 1973).

The kinetics of [^{125}I]enterotoxin binding to intestinal epithelial cells incubated at 37, 24, and 4 °C are shown in Figure 6. At all three temperatures the amount of label bound to the cells increased with time, reaching a maximum within 60 min. The rate of association and the maximum amount of enterotoxin binding appeared to be temperature dependent. The maximum number of molecules bound per cell under the conditions described (Figure 6) at 37 °C was 1.35×10^6/cell, at 24 °C was 1.00×10^6/cell, and at 4 °C was 0.30×10^6/cell.

When the association data at 24 °C in Figure 6 were replotted as percent total sites remaining unoccupied at a given time, a biphasic curve resulted as shown in Figure 7. The fast and slow associating species probably represent the high- and low-affinity sites, respectively. Association rate constants for the fast ($t_{1/2}$ = 1.2 min) and the slow ($t_{1/2}$ = 17 min) species can be calculated (Table I). Rates for the fast species were calculated from data taken between 0.5 and 5 min while the rates for the slow species were calculated from data taken between 5 and 40 min. The plots of data from 37 and 4 °C (not shown) were similar in appearance to that given in Figure 7 for data at 24 °C. While the rate constants were temperature dependent, the half-times of association for the two species of sites apparently were not appreciably altered by temperature (Table I).

Extrapolation of the association rates for the fast species back to zero indicated that at 4, 24, and 37 °C, 14, 18, and

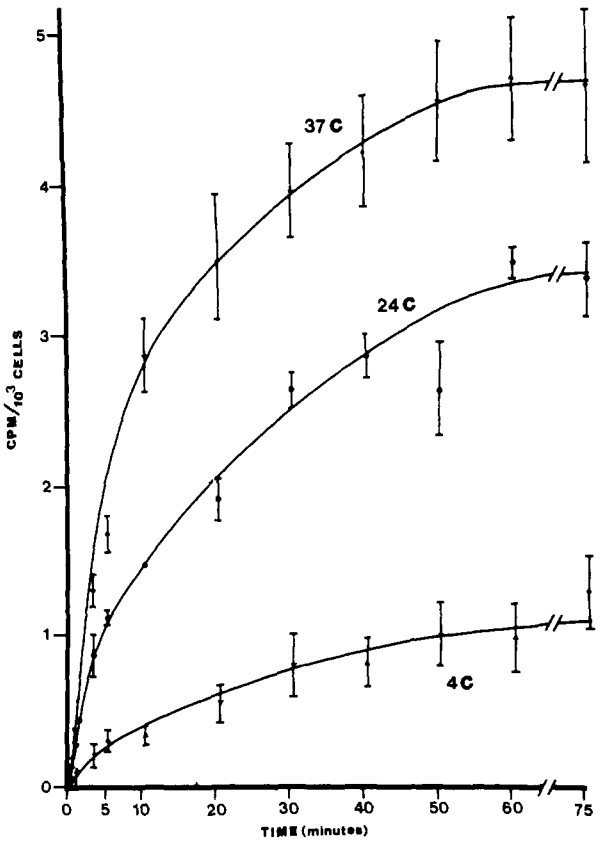

FIGURE 6: Specific binding of [^{125}I]enterotoxin to intestinal cells as a function of temperature and time. Duplicate tubes of cells (an average of 3.4×10^6, 3.13×10^6, and 2.97×10^6 cells/0.5 mL at 4, 24, and 37 °C, respectively) were incubated with 1 EU (0.5 μg) of [^{125}I]enterotoxin (2100 cpm/pmol) for the times and temperatures indicated. A third tube at each time and temperature point that had been pretreated for 60 min with 30 EU (8.6×10^{-10} M) of native enterotoxin was used to calculate nonspecific binding. Error bars show standard error of the mean, which if not shown was smaller than the symbol.

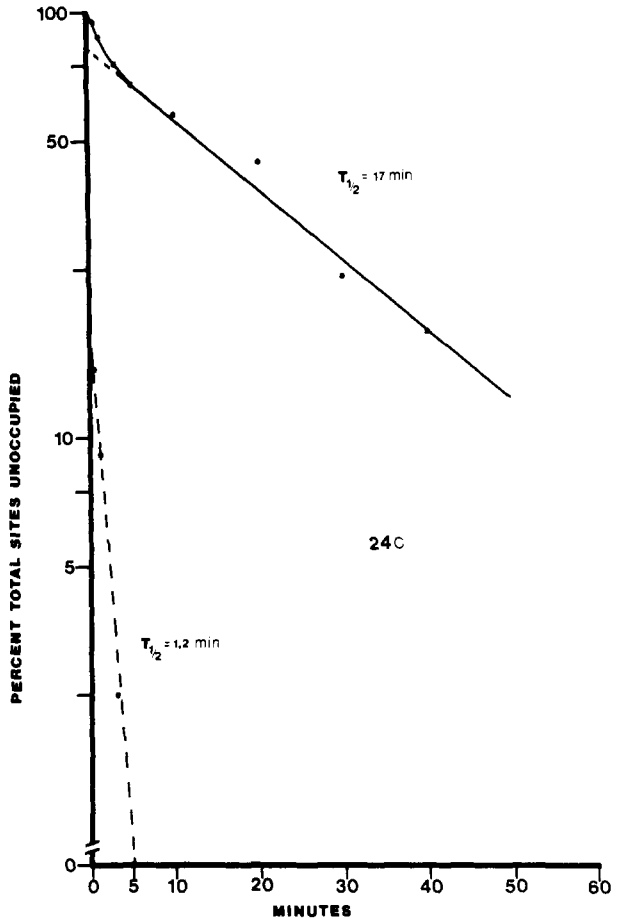

FIGURE 7: Replot of association data at 24 °C from Figure 6. Data are plotted as percent of total available sites remaining unoccupied at a given time. Half-time of association ($t_{1/2}$) for the fast species was calculated after correcting for binding to low-affinity (slow associating species) sites when extrapolated back to zero.

24%, respectively, of the total sites per cell were fast associating (high-affinity) sites. The value at 24 °C (18%) was somewhat lower but comparable to the value obtained from Scatchard analysis which indicated that at 24 °C, 32% of the total sites per cell were comprised of the high-affinity species.

By use of the apparent dissociation constants and the forward rate constants given in Table I, dissociation rate constants can be calculated, $k_{-1} = K_D \times k_1$. At 24 °C, $k_{-1} = 1.05 \times 10^{-3}$ and 9.27×10^{-3} s^{-1} for the high- and low-affinity sites, respectively. Attempts were made to measure the dissociation rate constant directly at 24 °C. Surprisingly, dissociation of bound label occurred very slowly. Dissociation, in the presence of native enterotoxin or when measured by the membrane filter method, occurred at a rate with a half-time of 125 ± 16 min (range 100–156 min). The average dissociation rate constant for these experiments was 9.2×10^{-5} s^{-1}, which is 2 orders of magnitude lower than the value calculated from $K_D \times k_1$. Furthermore, the observed dissociation was at a constant rate, which was not expected because of the existence of high- and low-affinity sites. The addition of chaotropic salts such as 0.5 M LiCl and 0.5 M KCNS had no augmenting effect upon the rate of dissociation in the membrane filter system.

The effects of proteolytic enzymes on binding are depicted in Table II. When cells were treated for 5 min with trypsin or Pronase prior to addition of [^{125}I]enterotoxin, the cells' capacity to bind enterotoxin was reduced by nearly 70% by trypsin and totally eradicated by Pronase. When cells that had been saturated with [^{125}I]enterotoxin were treated for 5 min with trypsin or Pronase, no release of counts was noted.

Table II: Effects of Trypsin and Pronase on Binding of *C. perfringens* [^{125}I]Enterotoxin

treatment	cpm bound[a]
control	41 200
Before Binding[b]	
trypsin	12 900
Pronase	0
After Binding[c]	
trypsin	38 900
Pronase	41 400

[a] Values given represent specific binding (total counts bound minus counts bound after pretreatment with an excess of native enterotoxin) at 24 °C. [b] Cells (3×10^6 in 0.5 mL) were treated at 24 °C for 5 min with trypsin (0.4 mg/mL) or Pronase (0.2 mg/mL) followed by two washes with buffer containing calf serum and two washes with normal buffer prior to addition of 7.0 EU of [^{125}I]enterotoxin. [c] After 1 h of binding, cells were washed and then treated for 5 min with trypsin (0.4 mg/mL) or Pronase (0.2 mg/mL).

The results of experiments to determine the effects of heat inactivation of the enterotoxin on its binding properties are given in Table III. Pretreatment of cells with active native enterotoxin caused inhibition of specific binding as expected (see Figure 2). No significant effect upon binding was noted when cells were pretreated with heat-inactivated (60 °C, 10 min) native enterotoxin. For confirmation of the loss of specific

Table III: Effect of Heat Inactivation[a] of Enterotoxin on Specific[b] Binding Properties

n	pretreatment	[125I]entero-toxin (EU)	cpm bound/10^6 cells[c]	sp binding (cpm/10^6 cells)
4	none (control)	2	4300 ± 358	3431
4	native toxin	2	869 ± 139	0
8	native toxin (HI)	2	3839 ± 71[d]	2970
8	none (control)	2 (HI)	1326 ± 96	0
8	native toxin	2 (HI)	1390 ± 137	
6	none (control)	4 (HI)	3099 ± 149	0
6	native toxin	4 (HI)	2815 ± 334[d]	

[a] Enterotoxin was heat inactivated (HI) for 10 min at 60 °C. [b] Specific binding refers to that binding which can be inhibited by pretreatment with native enterotoxin. [c] Values are means ± standard errors of the mean. [d] Difference from control value is not significant ($p > 0.05$).

Table IV: Binding of [125I]Enterotoxin[a] to Rabbit Intestinal Cells and Tissue Homogenates from the Liver, Kidney, and Brain

cell type	n	cpm bound/mg of cell protein	molecules of enterotoxin/mg of cell protein[b]
intestine	16	30 595 ± 2545	1.07×10^{13}
liver	13	26 655 ± 1615[c]	0.93×10^{13}
kidney	18	31 531 ± 1288[c]	1.10×10^{13}
brain	16	391 ± 427	

[a] Serial dilutions of cells or tissue homogenates (1×, 0.5×, 0.25×) were treated with 2 EU (1 μg or 5.7×10^{-11} M in 0.5 mL) of [125I]enterotoxin for 60 min at 24 °C, and the calculated values were summed. [b] Values are means ± standard error of the mean. [c] Difference from value for the intestine is not significant ($p > 0.05$).

binding activity through heat inactivation, [125I]enterotoxin was heat inactivated prior to addition to cells. Slightly more counts bound to cells treated with heat-inactivated [125I]-enterotoxin (1326 cpm) than bound to cells pretreated with native enterotoxin prior to exposure to active [125I]enterotoxin (869 cpm). However, the 1326 cpm is taken to represent nonspecific binding because pretreatment of cells with active native enterotoxin had no inhibitory effect upon the amount of counts bound to cells when treated with heat inactivated [125I]enterotoxin. When the dose of heat-inactivated [125I]-enterotoxin was doubled, the amount of nonspecific binding was doubled while specific binding remained at zero.

Table IV gives the results of binding studies done with intestinal cells and tissue homogenates from the liver, kidney, and brain. A fixed excess of [125I]enterotoxin was added to decreasing amounts of cells or homogenate to assure saturation of available sites so that maximum binding ability could be compared for each tissue. Amounts of [125I]enterotoxin bound per milligram of cell protein by liver and kidney cells were not significantly different from amounts bound by intestinal cells. It appears that the binding sites for enterotoxin are found in all three cell types at nearly equivalent densities per milligram of cell protein.

Brain tissue bound no significant amounts of enterotoxin. The possibility was considered that some inhibitory or competitive binding substance could have been released from the brain homogenate into the suspending buffer, thereby preventing binding of enterotoxin to the cells. The supernatants from brain homogenate binding experiments were collected and added to fresh preparations of intestinal cells to check this possibility. The amount of binding to the intestinal cells was identical with that for enterotoxin that had not been exposed to the brain homogenate preparations.

Discussion

This report provides data which suggest that *C. perfringens* [125I]enterotoxin binds to isolated intestinal epithelial cells and tissue homogenates from liver and kidney but not the brain of rabbits. Studies done with the intestinal cells indicate that the binding is a function of cell concentration, [125I]enterotoxin concentration, and temperature. The binding of the labeled enterotoxin is taken to be specific in that it is inhibitable by pretreatment with native enterotoxin. Furthermore, heat inactivation, which destroys biological activity, also destroyed binding activity.

The intestinal cells appear to have two classes of binding sites, which is in agreement with results reported previously (McDonel & McClane, 1979) for binding of [125I]enterotoxin to Vero (African green monkey kidney) cells. Further comparison with the Vero cell model system must be made with caution at this point since the temperature of incubation in the Vero cell system was 37 °C while most of the experiments done in this study were performed at 24 °C. The calculated density of total sites/cell was lower in Vero cells (1.30×10^6/cell) at 37 °C than in the intestinal cells (1.97×10^6/cell) at 24 °C. The distribution of high- and low-affinity sites was comparable in Vero and intestinal cells (40:60% high-/low-affinity sites, Vero cells; 32:68% high-/low-affinity sites, intestinal cells).

How sensitivity of Vero cells to the enterotoxin compares to that of the epithelial cells is not known at this time. It has been demonstrated in the Vero cell system that reducing by 10-fold the binding capacity of cells reduces the biological response to the enterotoxin by an equivalent amount (McDonel & McClane, 1979). Furthermore, when the concentration of free enterotoxin required for a half-maximal response is equated with the dissociation constant (K_D) (Cuatrecasas & Hollenberg, 1976), the relationship between binding and biological activity is further confirmed. A half-maximal inhibition of protein synthesis in Vero cells (McClane & McDonel, 1979) was accomplished by exposure to 7.27×10^{-8} M enterotoxin while the calculated K_D from binding studies and Scatchard analysis was found to be 6.37×10^{-8} M for the predominant (low-affinity) species of binding sites. If the same relationship holds true in the intestinal cells, then it would be expected that they would be more sensitive to the enterotoxin. Dissociation constants at comparable temperatures are not available for both cell types at this time.

There is some disagreement in the literature as to the relationship between binding and biological actvity. Middlebrook et al. (1978) equated receptor site density to sensitivity in Vero cells (utilizing 125I-labeled diphtheria toxin) vs. HeLa cells. However, Chang & Neville (1978) demonstrated that receptor site density in several different cell types is not necessarily related to cell sensitivity or resistance to diphtheria toxin. Considerable work has been done with *C. perfringens* enterotoxin using intact intestinal sections, but much work has yet to be done with the isolated intestinal cells.

The binding of [125I]enterotoxin to intestinal cells was found to meet the criteria proposed (Cuatrecasas & Hollenberg, 1975; Cuatrecasas, 1974) for identification of a receptor. The binding was specific in that native enterotoxin competed for 90% of the total association between cells and the enterotoxin. It was saturable as seen in Figure 3, and the high-affinity of association was indicated by the calculated affinity constant ($K_a = 5.66 \times 10^7$ and 8.22×10^6 L/M for intestinal cells at 24 °C). Direct observation of dissociation indicated that binding appears to be reversible. However, the rate of dis-

sociation expected based on calculations from $k_{-1} = K_D \times k_1$ was not observed experimentally. The observed rate was nearly 2 orders of magnitude lower than the calculated value. Furthermore, a biphasic curve (fast and slow dissociation species) did not result from the dissociation data. Cassidy & Harshman (1976) reported that [^{125}I]-α-toxin (*Staphylococcus*) appears not to dissociate at all, which is in disagreement with the dissociation rate constant (9.25×10^{-3} s^{-1}) that they calculated from Scatchard analysis of association data. However, they did demonstrate that chaotropic salts (which favor disruption of hydrophobic interactions) cause rapid release of the tightly bound toxin. Chaotropic salts did not cause a release of *C. perfringens* [^{125}I]enterotoxin from intestinal cells. We believe that the discrepancy between calculated and observed rates of dissociation and inaccessibility of the bound enterotoxin to release by chaotropic salts may be due to the nature of the enterotoxin–membrane interaction.

It is believed that the enterotoxin acts directly upon the cell membrane (McDonel et al., 1978; McClane & McDonel, 1979, 1980; McDonel, 1979). One hypothesis we propose is that the enterotoxin, after binding to its receptor, becomes inserted into the membrane or causes some configurational change in the membrane which results in the enterotoxin becoming "trapped". The decrease in total molecules bound by the cells with a decrease in temperature may reflect the importance of changes with temperature of membrane surface structure and configuration. Some membrane alterations that have been noted (McDonel et al., 1978), as well as the possible temperature dependence of availability of binding sites, may indicate the involvement of lipid in binding or structural damage to the membrane. However, the failure of the enterotoxin to bind at all to brain tissue, which has a high lipid content, casts doubt on this likelihood.

Dissociation from the receptor may occur before, during, or after the insertion step. The observed rate of release may represent a process that is the reverse of the membrane insertion step, not dissociation from the receptor. The release into the medium may be determined by the development of localized membrane damage (McClane & McDonel, 1980), resulting from the enterotoxin's presence. Therefore, the observed rate of release would be measuring a step beyond the receptor dissociation process. If the receptor were available as a molecule isolated from the cell membrane, then enterotoxin dissociation from the receptor could be studied directly. We currently are attempting to isolate an enterotoxin receptor molecule from intestinal cell brush border membranes for the purpose of conducting these kinds of studies.

It was demonstrated that trypsin and Pronase treatment of cells greatly reduces the level of binding of enterotoxin. These enzymes may reduce the number of binding sites or alter the membrane surface to mask existing sites. We have observed that Vero cells harvested by rubber policeman from monolayers bind 2 times as much enterotoxin as ones harvested by trypsinization (J. L. McDonel, unpublished observation). Of particular interest is the observation that bound enterotoxin is not released by trypsin or Pronase treatment. If these proteases degrade the binding sites, then bound enterotoxin should be released by protease treatment. The enterotoxin is digested by Pronase but not trypsin (Duncan & Strong, 1969; Hauschild & Hilsheimer, 1971), yet Pronase treatment did not cause a release of counts from the cells. Further work must be done to clarify the events at the cell membrane surface that are occurring when the enterotoxin interacts with its receptor.

At this time we believe that the enterotoxin is binding to a receptor, followed by insertion into the membrane which results in a breakdown in membrane structure and function. It is believed that the enterotoxin is a spore coat structural protein that is normally inserted into the bacterial spore coat (Frieben & Duncan, 1973, 1975). For this reason, the structure of the enterotoxin molecule may be conducive to insertion into the eukaryotic cell membrane but deleterious to its normal balance of structural components.

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