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Interaction of Diphtheria Toxin with Phosphorylated Molecules

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The binding of diphtheria toxin to ^{125}I-labeled cell surface glycoproteins from hamster thymocytes was shown to be inhibited by nucleotides. The relative effectiveness of the nucleotides (at 5 mM) was found to be thymidine triphosphate > adenosine triphosphate > guanosine triphosphate > uridine triphosphate > cytidine triphosphate. When adenine-containing compounds were used, the relative effectiveness was determined to be adenosine tetraphosphate > adenosine triphosphate > adenosine diphosphate > adenosine monophosphate. In addition, tetrapolyphosphate, tripolyphosphate, inositol hexaphosphate (phytic acid), and the highly phosphorylated proteins casein and phosvitin were also shown to be potent inhibitors of the binding of diphtheria toxin to ^{125}I-labeled cell surface glycoproteins. Diphtheria toxin was shown to bind directly to ^{125}I-casein; this binding was also inhibited by the highly phosphorylated compounds and was decreased by pretreatment of the ^{125}I-casein with alkaline phosphatase. These results suggest that diphtheria toxin binds to regions of high phosphate density and raise the possibility that the site on the cell surface glycoproteins to which diphtheria toxin binds might be polyanionic in nature.

Diphtheria toxin (DT) is a 62,000-dalton bacterial toxin that is cleaved by limited proteolysis into its two polypeptide fragments, fragment A (21,000 daltons) and fragment B (40,000 daltons), which remain linked to each other by a disulfide bond (4, 9).

The mechanism of action of the toxin on susceptible cells is believed to involve: (i) binding, via the B fragment, to cell surface receptors, (ii) translocation of the A fragment into the cytoplasm, and (iii) inhibition of protein synthesis by adenosine diphosphate (ADP) ribosylation of elongation factor 2, a process catalyzed by the enzymatic A fragment. Although the details on the ADP ribosylation of elongation factor 2 are well understood, less is known about the interaction between the toxin and its receptor, the nature of the receptor, and the mechanism of translocation.

The interaction between DT and its cell receptor has been studied recently by Middlebrook and co-workers (6, 7). They demonstrated that exogenous nucleotides protected Vero cells from the lethal effects of DT and that the nucleotides also inhibited the binding of ^{125}I-labeled DT to these cells. They concluded that the nucleotides exerted their effects by binding to the toxin receptor on cells since they were unable to demonstrate specific binding of nucleotides to DT (6).

We recently reported the detection of DT-binding cell surface glycoproteins from guinea pig lymph node cells (13) and from hamster thymocytes (12). The binding of DT to these cell surface glycoproteins was shown to be dependent on the presence of a functional B fragment (12, 13). The requirement for a functional B fragment is a property that these DT-binding cell surface glycoproteins share with the toxin receptor on cells (5, 7, 17).

We have now extended our studies to investigate the biochemical characteristics of the interaction of DT with the cell surface glycoproteins. In the present communication we report that nucleotides also inhibit the binding of DT to iodinated cell surface glycoproteins from hamster thymocytes, the degree of inhibition being related to the number of phosphate groups. This binding is also inhibited by casein and phosvitin, two proteins with high densities of phosphate groups. Furthermore, we report that DT interacts directly with ^{125}I-casein, at least in part via phosphate residues on the casein. All of these observations suggest that nucleotides and other polyphosphorylated compounds exert their effects not by interacting with cellular surface components, but rather by interacting with the toxin molecule itself.

MATERIALS AND METHODS

Materials. The nucleotides cytidine triphosphate, guanosine triphosphate, and uridine triphosphate were

purchased from PL Biochemicals, Inc., Milwaukee, Wis. All other nucleotides, sugar phosphates, phosphorylated amino acids, and polyphosphates were obtained from Sigma Chemical Co., St. Louis, Mo. Inositol hexasulfate was from Calbiochem, La Jolla, Calif. All compounds were sodium salts except for tetrapolyphosphate, which was the ammonium salt, and inositol hexasulfate, which was the potassium salt. Phosvitin and ovalbumin (grade VI) were also from Sigma Chemical Co. Casein was obtained from Difco Laboratories, Detroit, Mich., and aggregates were removed from its solutions by centrifugation at 100,000 × *g* for 1 h. Alkaline phosphatase (*Escherichia coli*) (EC 3.1.3.1) was purchased from Worthington Biochemicals Corp., Freehold, N.J.

Toxins, antisera, and other reagents. Partially purified DT was purchased from Connaught Laboratories, Ontario, Canada (lot 343) and was further purified by chromatography on DE-52 cellulose and ammonium sulfate fractionation according to published methods (11). The purified DT yielded a single protein band at 62,000 daltons on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in the absence of reducing agents. In the presence of 2-mercaptoethanol, SDS-polyacrylamide gel electrophoresis revealed that less than 10% of the molecules had undergone limited proteolysis. Crude diphtheria toxoid was a gift from P. Forsyth, Texas State Health Laboratories, Austin, and was further purified by ammonium sulfate fractionation and ion-exchange chromatography on DE-52 cellulose; the purified toxoid yielded a single protein band on analysis by SDS-polyacrylamide gel electrophoresis.

Antiserum to DT was produced in New Zealand white rabbits by hyperimmunization with purified toxoid. The resulting antiserum reacted with diphtheria toxoid as well as with DT, as determined by Ouchterlony analysis.

All of the phosphorylated compounds, chelating agents, and phosphoproteins utilized were prepared in a minimal volume of 0.1 M tris(hydroxymethyl)aminomethane(Tris)-hydrochloride (pH 7.4), the pH was adjusted with either 1 N HCl or NaOH to pH 7.4, and the solutions were diluted to the desired concentrations with 0.1 M Tris-hydrochloride, pH 7.4. The concentrations of nucleotides were determined spectrophotometrically, and all other concentrations were based on the weight obtained. Casein was iodinated by the chloramine-T method (2).

Radioiodination and lysis of cells. Thymus cells were obtained from normal female MHA or CB hamsters (Charles River Lakeview Breeding Laboratories, Newfield, N.J.). Single cell suspensions, 2×10^8 cells per 2 ml of phosphate-buffered saline (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2), were iodinated by the lactoperoxidase method, as previously described (13). The iodinated cells were washed in an excess of phosphate-buffered saline and lysed in 0.5% Nonidet P-40 (NP-40) (Shell Oil Co.) in Tris-buffered saline (TBS; 0.01 M Tris-hydrochloride, 0.15 M NaCl, 0.02% NaN_3, pH 7.4) at a ratio of 5×10^7 cells per ml of 0.5% NP-40 for 15 min (4°C). Nuclei were removed by centrifugation, and the supernatant fraction was dialyzed for 3 h against TBS at 4°C.

Preparation of the glycoprotein fraction. The dialyzed cell lysates were fractionated by lentil lectin-Sepharose affinity chromatography as previously described (13). After extensive washing of the lentil lectin-Sepharose columns with TBS–bovine serum albumin (BSA; 0.1 mg/ml)–0.1% NP-40 buffer, the adherent ^{125}I-glycoprotein fraction was eluted with 0.2 M α-methylmannoside in the same buffer. The eluted glycoprotein fraction was concentrated to the original volume of the ^{125}I-labeled cell lysate by vacuum dialysis against TBS. The entire procedure was performed at 4°C. The ^{125}I-glycoprotein fraction obtained was utilized for the detection of DT-binding glycoproteins by the immunoprecipitation assay (13).

Immunoprecipitations. Immunoprecipitation of the ^{125}I-glycoprotein fraction was performed as previously described (13). To 0.9-ml samples of the ^{125}I-glycoprotein fraction either 0.1 ml of 0.1 M Tris-hydrochloride, pH 7.4 (control) or 0.1 ml of the compound being tested (made in 0.1 M Tris-hydrochloride, pH 7.4, as described above) was added. This mixture was then incubated with DT for 1 h at 37°C, which was followed by incubation with antiserum to DT for 1 h at 4°C. The resulting immune complexes were adsorbed onto fixed *Staphylococcus aureus* bearing protein A. The immune precipitates complexed to the bacterial pellets were washed three times with TBS–BSA–NP-40 buffer. The radioactivity associated with these pellets was determined by counting in a gamma spectrometer. Results of representative experiments are presented below. Each experiment was performed a minimum of three times. The addition of 10 mM inositol hexaphosphate or adenosine triphosphate (ATP) had no effect on the immunoprecipitation system as tested with ^{125}I-labeled DT and antiserum to DT followed by *S. aureus*.

For the detection of binding of DT to ^{125}I-casein, an immunoprecipitation assay system was also employed. The system was the same as the one described above except that ^{125}I-casein (in TBS–BSA–NP-40 buffer) was substituted for the ^{125}I-glycoprotein fraction.

SDS-polyacrylamide gel electrophoresis. The radiolabeled proteins isolated by immunoprecipitation were dissociated, reduced, and electrophoresed on SDS–5% polyacrylamide gels by the method of Shapiro et al. (14), as previously described (13).

RESULTS

We have previously developed and described a system for detecting DT-binding cell surface glycoproteins from guinea pig lymph node cells (13) and from hamster thymocytes (12). The latter were chosen for the present study to characterize further the interaction between the toxin and the DT-binding glycoproteins.

Effect of adenine nucleotides on DT binding to cell surface glycoproteins. Adenine-containing nucleotides have been shown to protect cells from killing by DT and to inhibit the binding of radiolabeled DT to cells (6, 7) and to plasma membrane preparations (3); thus, their effects on the binding of DT to radioiodinated cell surface glycoproteins were investigated first (Table 1). All of the adenine-containing nucleo-

TABLE 1. *Effects of nucleotides, polyphosphates, and chelating agents on the binding of DT to iodinated cell surface glycoproteins*[a]

Compound added	Amt of radioactivity precipitated (% of control) when concn of compound was:[b]		
	1 mM	5 mM	10 mM
Adenosine tetraphosphate	49	33	
ATP	67	44	30
ADP		58	
Adenosine monophosphate	82	71	
Nicotinamide adenine dinucleotide	76	80	
Thymidine triphosphate		43	
Guanosine triphosphate		60	
Uridine triphosphate		77	
Cytidine triphosphate		83	
Tetrapolyphosphate	68	52	
Tripolyphosphate	99	63	
Pyrophosphate	115	82	
Orthophosphate			101
Ethylenediaminetetraacetate		99	103
EGTA[c]		102	114

[a] The compound to be tested was added to samples of the iodinated glycoprotein fraction (2×10^7 cell equivalents). DT (30 μg) and then antiserum to DT (40 μl, a quantity sufficient to precipitate 30 μg of DT) were added, and the immune complexes were processed as described in the text.

[b] The radioactivity precipitated in the control reaction mixture was 35,820 cpm; all values in this table are reported relative to this control value. Substitution of diphtheria toxoid for DT resulted in 4,130 cpm precipitated.

[c] EGTA, Ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid.

tides exhibited a dose-dependent inhibitory effect on the DT-glycoprotein binding system. Adenosine tetraphosphate was the most potent inhibitor, followed by ATP, ADP, and adenosine monophosphate. Nicotinamide adenine dinucleotide, which is a substrate for the reaction catalyzed by fragment A of DT and could be considered an analog of both ADP and adenosine monophosphate, was less inhibitory than ADP and adenosine monophosphate (Table 1).

These results demonstrated an effective decrease in the total radioactivity immunoprecipitated from the cell surface glycoprotein fraction by the adenine nucleotides. That the decrease observed was specific for the DT-binding glycoproteins was demonstrated by analysis of these immune precipitates by SDS-5% polyacrylamide gel electrophoresis. As Fig. 1 shows, increasing the concentrations of adenosine tetraphosphate and ATP resulted in proportional decreases in the size of the major ^{125}I-glycoprotein peak obtained. The percent inhibition calculated from the amount of radioactivity under the major peak (data not shown) was similar to the percent inhibition calculated from the total radioactivity immunoprecipitated (Table 1).

Effects of other nucleotide triphosphates and of polyphosphates. Middlebrook et al. (6)

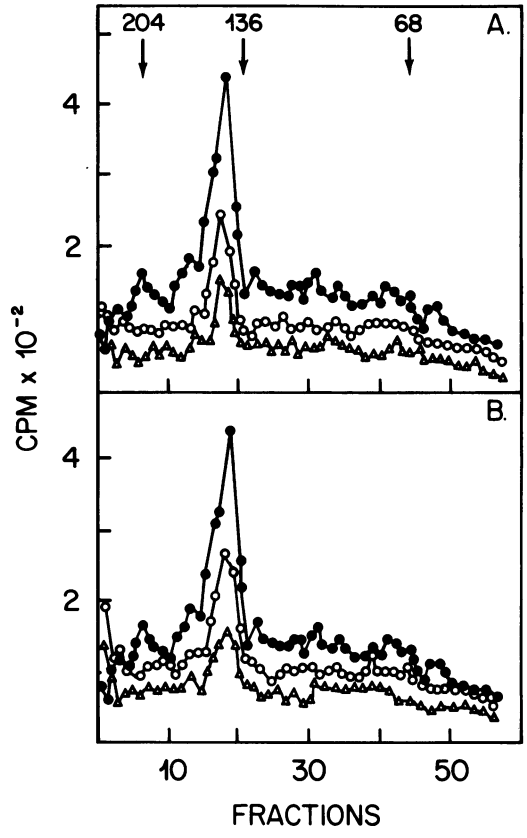

FIG. 1. *SDS-polyacrylamide gel electrophoresis of DT-binding glycoproteins. This analysis was performed on immune precipitates of the ^{125}I-glycoprotein fraction shown in Table 1. (A) Symbols: ●, control (DT plus antiserum to DT); ○ and △, addition of adenosine tetraphosphate (before DT plus antiserum to DT) at a concentration of 1 mM (○) or 5 mM (△). (B) Symbols: ●, control (DT plus antiserum to DT); ○ and △, addition of ATP (before DT plus antiserum to DT) at a concentration of 1 mM (○) or 5 mM (△). The immune precipitates were dissociated, reduced, and applied to SDS-5% polyacrylamide gels. The gels were electrophoresed at 7 mA/gel for 16 h. The gels were sliced, and each fraction was analyzed for radioactivity. The arrows indicate the molecular weights (×10³) of the internal ^{3}H-markers, which consisted of ^{3}H-labeled BSA monomer (68,000), cross-linked ^{3}H-labeled BSA dimer (136,000), and cross-linked ^{3}H-labeled BSA trimer (204,000).*

demonstrated that protection of cells from the cytotoxic effects of DT and the inhibition of binding of ^{125}I-labeled DT to cells were not limited to adenine-containing nucleotides and that other purine and pyrimidine triphosphates also had these properties. These same nucleotide triphosphates were tested for their possible effects on the binding of DT to radioiodinated cell surface glycoproteins. At a concentration of 5 mM, thymidine triphosphate was found to be as effective an inhibitor as the ATP; guanosine triphosphate was the next best inhibitor, followed by uridine triphosphate and cytidine triphosphate. (Table 1). Interestingly, this is the same relative order that was found by Middlebrook et al. (6) for the protective effect of the nucleotide triphosphates and for the inhibition of binding of radioactive DT to cells.

Next, the effects of inorganic polyphosphates were investigated. Tetrapolyphosphate at 5 mM was less inhibitory than adenosine tetraphosphate, ATP, and thymidine triphosphate, but more inhibitory than the other nucleotides tested (Table 1). Tripolyphosphate was less inhibitory than tetrapolyphosphate but more inhibitory than pyrophosphate. Orthophosphate (up to 10 mM) had no inhibitory effect (Table 1). Thus, the degree of inhibition was related to the chain length of the polyphosphate; this was similar to the pattern observed with the adenine nucleotides. This order of relative effectiveness was observed in all three experiments performed. This latter observation, that the degree of inhibition was dependent on the number of phosphate moieties per molecule, suggested that the mode of inhibition could be due to chelation of a cation involved in the binding of DT to cell surface glycoproteins. It is not likely that this is the case since inclusion of ethylenediaminetetraacetate or ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetracetic acid (5 or 10 mM) had no inhibitory effect (Table 1).

Effects of phosphorylated amino acids and phosphorylated sugars. The results obtained with phosphorylated nucleotides and with polyphosphates prompted us to investigate the effects of other phosphorylated compounds. Table 2 summarizes the results obtained. Phosphoserine, phosphothreonine, and mannose 6-phosphate had no inhibitory effect, and the effect of glucose 6-phosphate was only minimal. However, inositol hexaphosphate (phytic acid) had a dramatic inhibitory effect; at 5 mM it was as inhibitory as 5 mM adenosine tetraphosphate (Tables 1 and 2). In contrast, inositol hexasulfate at 5 mM was without effect.

Effects of phosphorylated proteins. The fact that the monophosphorylated amino acids and sugars tested did not inhibit the binding of DT to the cell surface glycoproteins but inositol hexaphosphate, which is highly phosphorylated, did inhibit binding suggested that areas of high phosphate density are important for the inhibition of the interaction of DT with the cell surface glycoproteins. Such areas of high phosphate density are naturally present in some phosphoproteins. Casein contains several (five to eight) phosphoserine residues, some of which are clustered in a SerP-*X*-SerP-SerP-SerP linear sequence (15). Phosvitin contains more than 100 phosphoserine residues, many of which are clustered in groups of six to eight contiguous phosphoserine residues (15). In contrast, ovalbumin contains only two phosphoserine residues, and these are located 20 residues apart (8). These three phosphoproteins were tested for their effects on DT binding to iodinated cell surface glycoproteins. Both casein and phosvitin greatly inhibited the binding, whereas ovalbumin had only a minimal effect (Table 2).

Interaction of DT with ^{125}I-casein. The experimental results obtained with the inhibitors suggested that either they were interacting with the iodinated cell surface glycoproteins and preventing toxin binding or they were binding to the toxin itself and preventing its binding to the cell surface glycoproteins. The latter possibility was tested directly by substituting ^{125}I-labeled casein for the iodinated cell surface glycoproteins in the immunoprecipitation system. That is, ^{125}I-casein was permitted to react with DT, and the amount bound was detected by immunoprecipitation with antiserum to DT. The results (Table 3) demonstrated that DT binds to casein, whereas diphtheria toxoid, which does not bind to the cell surface glycoproteins (12, 13), binds to a much lower extent (eight fold lower). Furthermore, the binding of DT to ^{125}I-casein was inhibited by inclusion of ATP in this system (Table 3). This latter result suggests, although indirectly, that ATP binds to DT and would explain the reported effect of ATP protection of cell killing by DT (6, 7). That ATP indeed binds to DT has been shown recently by S. Lory and R. J. Collier (personal communication), who used a flow dialysis technique.

The inefficient binding of diphtheria toxoid to casein and the inhibition of the binding of DT to casein by ATP suggested that casein was behaving in this system in a manner similar to the DT-binding cell surface glycoproteins. To evaluate these similarities further, some of the phosphorylated compounds tested for their effects on the binding of DT to radioiodinated cell surface glycoproteins (Tables 1 and 2) were also tested for their effects on the binding of DT to

TABLE 2. *Effects of phosphorylated amino acids, phosphorylated sugars, and phosphoproteins on the binding of DT to iodinated cell surface glycoproteins*[a]

Compound added	Amt of radioactivity precipitated (% of control) when concn of compound was:[b]					
	1 mM	5 mM	0.25 mg/ml	0.50 mg/ml	1.5 mg/ml	2.5 mg/ml
DL-Phosphoserine		97				
L-Phosphoserine		105				
D-Phosphoserine		101				
DL-Phosphothreonine		104				
Mannose 6-phosphate		95				
Glucose 6-phosphate		85				
Inositol hexaphosphate (phytic acid)	49	32				
Inositol hexasulfate		98				
Ovalbumin				89		87
Casein			46	36	24	
Phosvitin			57	53	50	

[a] The conditions of the assay were the same as those described in Table 1, footnote *a*.
[b] The radioactivity precipitated in the control reaction mixture was 77,050 cpm.

TABLE 3. *Specificity of the reaction of DT with iodinated casein*[a]

Amt of ^{125}I-casein (μg)	Amt of DT (μg)	Amt of toxoid (μg)	Concn of ATP (mM)	Amt immunoprecipitated (cpm)
30	30			179,350
30		30		22,130
30	30		10	75,880
30		30	10	20,890

[a] ^{125}I-casein (30 μg; 2.5×10^6 cpm) was incubated with either DT or diphtheria toxoid; this was followed by the addition of antiserum to DT (40 μl, a quantity sufficient to precipitate 30 μg of DT or toxoid), and the immune complexes were processed as described in the text. When ATP was included in the assay, it was added before the DT or the diphtheria toxoid.

^{125}I-casein. The results of these experiments are shown in Table 4. Adenosine tetraphosphate, ATP, tetrapolyphosphate, and tripolyphosphate were considerably inhibitory. These compounds inhibited the binding of DT to ^{125}I-casein to approximately the same extent they inhibited the binding of DT to ^{125}I-labeled cell surface glycoproteins (Tables 1 and 4). DL-Phosphoserine and glucose 6-phosphate had a minimal inhibitory effect, whereas DL-phosphothreonine and mannose 6-phosphate had no effect (Table 4). Inositol hexaphosphate was a potent inhibitor; at a concentration of 1 mM it inhibited the binding of DT to ^{125}I-casein by 75% (Table 4). In contrast, inositol hexasulfate at 1 mM was not inhibitory and at 5 mM was only 25% inhibitory (Table 4), suggesting that the inhibitory effect is phosphate specific. Phosphoproteins were also tested in this system. Ovalbumin had only a minimal effect, whereas phosvitin was a potent inhibitor and, as expected, unlabeled casein (25-fold excess) significantly decreased the binding of DT to ^{125}I-casein (Table 4).

TABLE 4. *Inhibition of binding of DT to iodinated casein by phosphorylated compounds and analogs*[a]

Compound added	Concn		Amt of radioactivity precipitated (% of control)[b]
	mM	mg/ml	
Adenosine tetraphosphate	5		33
ATP	5		51
Tetrapolyphosphate	5		33
Tripolyphosphate	5		36
DL-Phosphoserine	5		75
DL-Phosphothreonine	5		105
Mannose 6-phosphate	5		108
Glucose 6-phosphate	5		84
Inositol hexaphosphate	1		25
	5		21
Inositol hexasulfate	1		94
	5		73
Ovalbumin		0.5	89
Phosvitin		0.5	29
Casein		0.5	9

[a] The compounds tested were added to ^{125}I-casein (20 μg; 1.5×10^6 cpm), and the mixtures were incubated with DT (10 μg), followed by antiserum to DT (20 μl, a quantity sufficient to precipitate all of the toxin added), as described in the text.
[b] The radioactivity precipitated in the control reaction mixture was 64,080 cpm.

That DT might bind to the phosphate groups on the casein molecule was suggested by the results obtained when casein was treated with alkaline phosphatase. When ^{125}I-casein was incubated with alkaline phosphatase and subsequently reacted with DT plus antiserum to DT,

the amount of radioactivity immunoprecipitated was decreased by 66% as compared with a control sample which was incubated without the enzyme (Table 5). Since the inhibitory effect seen after alkaline phosphatase treatment could have been due to a conformational change caused by the removal of phosphates, the following experiment was performed. ^{125}I-casein was heat treated (100°C, 2 min) in 0.1% SDS and tested for its DT-binding ability. Casein treated in this manner was as reactive with DT as an untreated control sample (Table 5). These results imply that the conformation of the casein does not play a prominent role in the interaction with DT but the phosphate groups do. It is of interest to point out that the interaction between the DT-binding cell surface glycoproteins from hamster thymocytes and the toxin is also resistant to heat treatment in SDS (12); however, treatment of the iodinated cell surface glycoproteins with alkaline phosphatase did not result in a decrease in the amount of radioactivity immunoprecipitated (data not shown).

DISCUSSION

We demonstrated that the binding of DT to iodinated cell surface glycoproteins from hamster thymocytes is inhibited by adenosine tetraphosphate, ATP, other nucleotide triphosphates, and polyphosphates. The specificity of these inhibitors correlates well with that observed for the toxin receptor on cells, as determined by binding assays with radiolabeled DT and by cytotoxicity methods (6, 7). We also showed that two highly phosphorylated proteins, casein and phosvitin, and a highly phosphorylated small molecule, inositol hexaphosphate, also inhibit the interaction of DT with iodinated cell surface glycoproteins. Moreover, we demonstrated that DT directly binds to ^{125}I-casein and that this binding is also inhibited by the same phosphorylated compounds that inhibit the interaction of DT with the iodinated cell surface glycoproteins. The binding of DT to ^{125}I-casein is decreased if the casein is pretreated with alkaline phosphatase. These results, taken together, strongly suggest that DT is a polyphosphate-binding protein. In this context, it is of interest to point out that *Corynebacterium diphtheriae* synthesizes and accumulates intracellular polyphosphate (metachromatic) granules (1). The interaction, if any, between toxin and these granules would be of interest and might be physiologically important to the organism.

TABLE 5. *Effect of alkaline phosphatase and heat treatment of iodinated casein on the interaction of casein with DT*[a]

Treatment	Amt of radioactivity precipitated (cpm)	% of control
Expt 1		
Control	57,460	100
Alkaline phosphatase	19,500	34
Expt 2		
Control	49,920	100
Heat treatment	48,850	98

[a] In experiment 1, ^{125}I-casein (0.4 mg/ml in 0.1 M Tris-hydrochloride, pH 8; 0.25-ml fractions) was incubated at 37°C for 16 h without (control) or with alkaline phosphatase (1.2 U). At the end of this incubation period, alkaline phosphatase (1.2 U) was added to the control. Then 20 μl (5×10^5 cpm) from each of these reaction mixtures was diluted to 1 ml with TBS–BSA–NP-40 buffer and assayed for DT binding by using 10 μg of DT followed by 20 μl of antiserum to DT as described in the text. In experiment 2, ^{125}I-casein (0.4 mg/ml in 0.1 Tris-hydrochloride, pH 8; 0.25-ml fractions) either was not treated (control) or was made 0.1% in SDS and heated at 100°C for 2 min. Then 20 μl (5×10^5 cpm) from each of these reaction mixtures was diluted to 1 ml with TBS–BSA–NP-40 buffer and assayed for DT binding as described above.

Our results also suggest that the protection of mammalian cells from the lethal effects of DT by nucleotides reported by Middlebrook and coworkers (6, 7) is most likely due to the binding of these phosphorylated compounds to the toxin molecule and not due to their binding to the toxin receptor on cells, as was concluded by these authors (6).

The interaction between DT and polyphosphate is probably not due to a simple ionic interaction, since at neutral pH both the polyphosphate and the DT (pI of ~6, [10]) bear a net negative charge. Thus, a specific interaction would have to take place between the polyphosphate and a positively charged region on the DT molecule. Based on the existence of such a polyphosphate-binding site on the DT molecule, the inhibition of DT binding to cell surface glycoproteins by highly phosphorylated moieties could be explained by either of the following hypotheses: (i) the positively charged polyphosphate-binding site on the DT is distinct from the cell surface glycoprotein-binding site on the DT so that the binding at the former site modulates the binding at the latter site; or (ii) the positively charged polyphosphate-binding site on the DT is the same as the cell surface glycoprotein binding site on the DT. The second hypothesis is the simplest and an attractive one in light of the evidence presented by Pappenheimer (10), which suggests that there is a positively charged region on the DT located in the 15,000- to 17,000-

dalton carboxy-terminal end of the B fragment. This region is believed to be the receptor binding area of the B fragment since it is present in DT and deleted in CRM 45 protein, a protein that is enzymatically active, is nontoxic, and apparently does not bind to cell surface receptors (9, 11, 16). The inhibition of DT-receptor interaction by polyphosphates would thus be a result of a competition between the polyphosphate and the toxin receptor for the carboxy-terminal positively charged binding site on the toxin molecule. If this hypothesis is correct, our results would also imply that the cell surface glycoproteins which bind to DT contain negatively charged groups responsible for this interaction. In this regard, it is interesting to note that treatment of the iodinated cell surface glycoproteins with alkaline phosphatase resulted in no decrease in the amount of radioactivity immunoprecipitated with DT. This result suggests that either the polyanionic moieties are phosphate groups present in an alkaline phosphatase-resistant linkage or the polyanionic groups responsible for the interaction are not phosphate groups. They may be sulfate or carboxyl moieties. Therefore, the chemical nature of the putative polyanionic groups on the DT-binding cell surface glycoproteins remains to be elucidated.

This hitherto undescribed biochemical property of DT, the binding to phosphorylated molecules, should aid in the study of the biology of DT. On the other hand, this finding should also serve as a cautionary note to those investigating the biochemical nature of the biological cell surface receptor for DT.

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