

Diphtheria toxin has the properties of a lectin

[concanavalin A/wheat germ agglutinin/cell surface receptors/ovalbumin (oligosaccharides) glycopeptide/cytotoxicity]

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ABSTRACT The inhibition of protein synthesis in Chinese hamster V79 cells by diphtheria toxin is antagonized by the lectins concanavalin A, succinylated concanavalin A, and wheat germ agglutinin but not by *Proteus vulgaris* phytohemagglutinin or abrus agglutinin. The effects of concanavalin A and wheat germ agglutinin are reversed by methyl α -mannoside and *N*-acetylglucosamine, respectively. The inhibition of diphtheria toxin as a function of concanavalin A concentration fits a model of competitive inhibition with an apparent dissociation constant for concanavalin A of 3×10^{-8} M. These results suggest that the diphtheria toxin receptor may be an oligosaccharide. To test this hypothesis, we screened several oligosaccharides for the ability to inhibit diphtheria toxin. The cell wall polysaccharide of *Salmonella cholerae suis* and the ovalbumin glycopeptide were effective inhibitors. These studies suggest that diphtheria toxin may have the oligosaccharide binding properties of a lectin with specificity for *N*-acetylglucosamine and mannose.

Diphtheria toxin (DT), a protein of molecular weight 63,000, kills animal cells by a three-step process (1, 2). First, the toxin binds to the cell surface; second, a 24,000 molecular weight fragment of the toxin is somehow transferred to the cytoplasm; and third, this fragment catalyzes the transfer of ADP-ribose from NAD^+ to elongation factor II, rendering it inactive. Nothing is known about the chemical nature of the DT receptor. Several plant and bacterial toxins poison animal cells by a general sequence of events similar to that of diphtheria intoxication—e.g., toxin binding to a cell surface receptor, an internalization or activation step, and, finally, expression of a catalytic activity that incapacitates the cell. These include cholera toxin (3), tetanus toxin (4), *Pseudomonas aeruginosa* toxin (5), abrin (6), and ricin (6).

One generalization concerning the nature of toxin cell surface receptors is that they are carbohydrate-containing molecules, either glycolipids or glycoproteins. Cholera toxin and tetanus toxin bind to ganglioside sugar residues (7, 8) whereas abrin and ricin bind to galactose-containing oligosaccharides (6). These toxic proteins exhibit carbohydrate-binding properties similar to lectins but without the agglutination activity of lectins. By analogy, these observations suggest that DT might also bind to a carbohydrate-containing receptor.

Attempts to test this hypothesis by inhibiting DT with various monosaccharides have been unsuccessful; however, the toxin may be specific for a more complex oligosaccharide sequence. If this were true, the toxin might be inhibited by lectins that could compete with the toxin for surface receptors. If such inhibitors were found, their carbohydrate-binding specificities might also help indicate the sort of oligosaccharides with which DT may interact. The results of our experiments to test these ideas are reported here.

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MATERIALS AND METHODS

Diphtheria toxin (Connaught Laboratories, Willowdale, Ontario, Canada) was provided by N. Kaplan. Two different lots were used: lot D297 was used without further purification; lot D298 was further purified by DE-52 chromatography and showed a single band on polyacrylamide gel electrophoresis. Although lot D297 was more active than lot D298, both gave similar results in these studies.

Concanavalin A (Con A), agarose-bound Con A, and wheat germ agglutinin (WGA) were obtained from Calbiochem (San Diego, CA). *Proteus vulgaris* phytohemagglutinin was purchased from Miles Laboratories (Elkhart, IN). Abrus agglutinin was purified from *Abrus precatorius* seeds by the method of Wei *et al.* (9) and succinyl Con A (Suc-Con A) was prepared by the procedure of Gunther *et al.* (10). The cell wall polysaccharides of *Salmonella cholerae suis* 5210, *Saccharomyces cerevisiae*, and *Kluyveromyces lactis* were provided by S. Sarkar; these polysaccharides were extensively dialyzed before use. Ovalbumin was purchased from Sigma Chemical Corporation (St. Louis, MO), and ovalbumin glycopeptide was prepared by Pronase digestion of ovalbumin by the procedure of Huang *et al.* (11). The lectins were tested for activity by hemagglutination.

Chinese hamster V79 cells were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.02 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate buffer (pH 7.4), kanamycin and neomycin each at 50 $\mu\text{g}/\text{ml}$, and Fungizone at 2.5 $\mu\text{g}/\text{ml}$. Protein synthesis was measured by incorporation of [^{35}S]-methionine into trichloroacetic acid-insoluble material. At 24 hr before an experiment, the cells were plated in 24-well Linbro plates at 1.1×10^5 cells per cm^2 in modified Eagle's medium containing 5% dialyzed fetal bovine serum and $1/10$ the normal amount of methionine. When a lectin that bound glucose was tested, medium lacking glucose but supplemented with galactose at 5 g/liter was used.

The standard assay for the inhibition of protein synthesis was initiated by addition of fresh medium containing DT with or without a potential antagonist. After a 2-hr incubation at 37°, 0.1 μCi of [^{35}S]-methionine was added. One hour later, the cells were washed twice with 2 ml of phosphate-buffered saline containing L-methionine (1.0 mg/ml) and dissolved in 0.5 ml of 0.1% sodium dodecyl sulfate. Protein was then precipitated with 10% trichloroacetic acid and the precipitates were collected on filters, washed, dried, and assayed for radioactivity in a liquid scintillation counter. The response of cells to toxin was calculated by the equation of Ittelson and Gill (12): (c —

Abbreviations: DT, diphtheria toxin; Con A, concanavalin A; WGA, wheat germ agglutinin; Suc-Con A, succinyl Con A; GlcNAc, *N*-acetylglucosamine; MeMan, methyl α -mannoside.

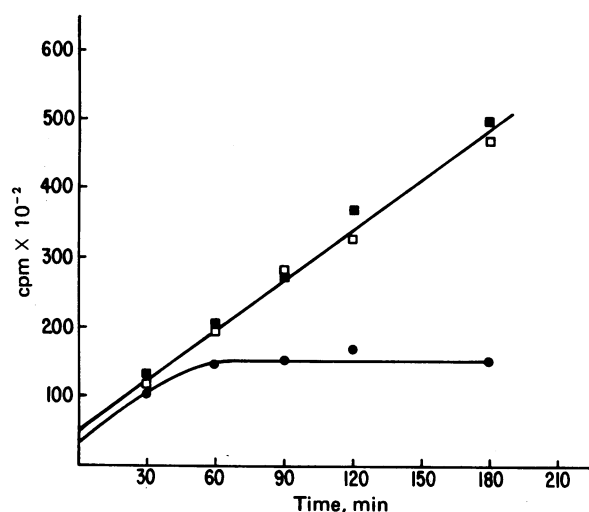


FIG. 1. Protein synthesis measured by incorporation of [35 S]-methionine into trichloroacetic acid-insoluble material. \square , Normal synthesis; \bullet , synthesis in the presence of $0.25 \mu\text{M}$ DT; \blacksquare , synthesis in the presence of $0.25 \mu\text{M}$ DT with cells pretreated for 15 min with Con A at $100 \mu\text{g/ml}$.

c'/c in which c is the counts per minute incorporated in the absence of toxin and c' is the counts per minute incorporated in the presence of toxin with or without antagonist. A Schild plot (12–15) was constructed to test for competitive inhibition of DT by Con A.

RESULTS

Effects of Lectins on DT. The effects of DT and DT plus Con A on the rate of protein synthesis are shown in Fig. 1. The cells were incubated for 15 min in medium containing Con A at $100 \mu\text{g/ml}$ and then DT was added to the cultures. The cells were completely protected at a toxin concentration that normally inhibited protein synthesis by 100% within 60 min. Identical results were obtained when DT and Con A were added simultaneously or when the cells were incubated with Con A for 15 min, washed in medium lacking Con A, and then treated with DT. When the cells were treated with DT 15 min prior to Con A addition, no protection was observed, suggesting that the protective effects of Con A involved the inhibition of an early step in the sequence of events involved in intoxication.

For further analysis of the effects of Con A and other lectins, we used the standard assay conditions described in *Materials and Methods*. A response of 1.0 corresponds to 100% inhibition of protein synthesis. Con A at $5 \mu\text{g/ml}$, Suc-Con A at $15 \mu\text{g/ml}$, and WGA at $6 \mu\text{g/ml}$ each inhibited the response of cells to 0.7 nM DT by about 50%. Within the concentration range used, these three lectins by themselves had no effect on protein synthesis during the 3-hr period of the assays. *P. vulgaris* phytohemagglutinin at concentrations approaching 1 mg/ml did not inhibit DT or protein synthesis. Abrus agglutinin alone at $30 \mu\text{g/ml}$ inhibited protein synthesis, but this effect was additive to the inhibition produced by DT, indicating that no antagonism of the toxin had occurred. Thus, lectins specific for mannosyl or *N*-acetylglucosaminyl oligosaccharides were good toxin inhibitors but those binding galactose or *N*-acetylglucosamine did not inhibit.

The response of cells to toxin in the presence of WGA could be restored to normal by including 50 mM *N*-acetylglucosamine (GlcNAc) in the medium during the assay. The effects of Con A and Suc-Con A were also reversed in the presence of 50 mM

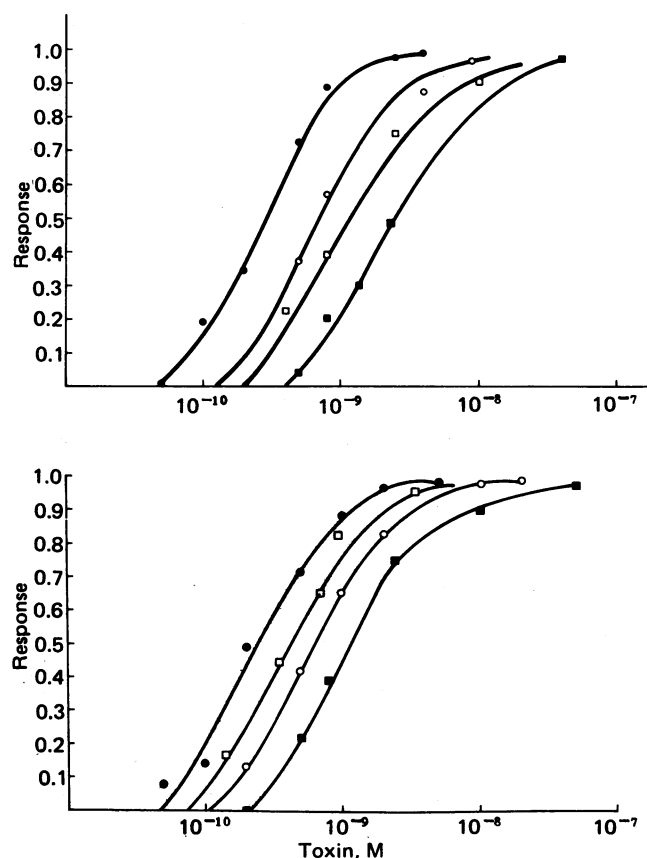


FIG. 2. Response of cells as a function of DT concentration in the presence of various lectins. A response of 1.0 corresponds to 100% inhibition of protein synthesis. (Upper) Effect of various concentrations of Con A: \bullet , none; \circ , $5 \mu\text{g/ml}$; \square , $10 \mu\text{g/ml}$; \blacksquare , $15 \mu\text{g/ml}$. (Lower) Effect of different lectins: \bullet , none; \square , WGA, $4 \mu\text{g/ml}$; \circ , Suc-Con A, $15 \mu\text{g/ml}$; \blacksquare , Con A, $10 \mu\text{g/ml}$.

methyl α -mannoside (MeMan), although at this concentration; MeMan itself decreased protein synthesis slightly. These experiments indicate that the carbohydrate-binding ability of each lectin is responsible for its antagonistic effects.

The effects of the inhibitory lectins on the response of cells as a function of DT concentration are shown in Fig. 2. The curves have the same shape and reach the same maxima in the presence or absence of inhibitors; the antagonist shifts the curve to higher toxin concentrations, where the inhibition is overcome. This behavior has been taken as presumptive evidence for competitive inhibition (13). To substantiate this suggestion, we constructed a Schild plot (Fig. 3) from the response curves of DT and a set of curves obtained at different Con A concentrations. This approach was used by Ittelson and Gill (12) to study competitive inhibition between DT and the nonlethal cross-reacting mutant protein 197. From a model for competitive inhibition, the following relationship was derived (15): $(T'/T) - 1 = I'/K_D$, in which T is the toxin concentration in the absence of inhibitor at a given response, T' is the concentration that gives the same response in the presence of inhibitor, I' is the concentration of inhibitor, and K_D is the apparent dissociation constant of the inhibitor. For competitive inhibition, a plot of $\log [(T'/T) - 1]$ versus $\log I'$ will be linear with a slope of 1 and an intercept of $-\log K_D$. This is the case in Fig. 3 for Con A; the slope is 1.2 and the apparent K_D is $3 \times 10^{-8} \text{ M}$.

The data in Figs. 2 and 3 could be explained if DT and the lectin compete for a cellular target or, alternatively, if the lectin reversibly interacts with DT to form an inactive complex (14).

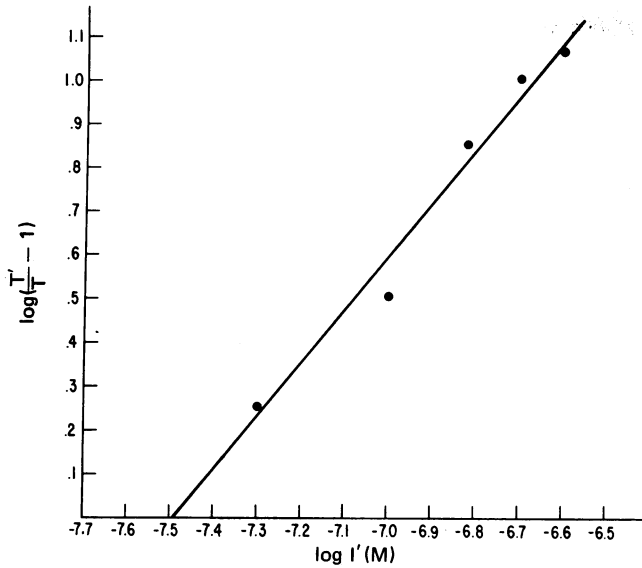


FIG. 3. Schild plot for the inhibition of DT by Con A, constructed from a set of dose-response curves such as in Fig. 2 Upper. T is the concentration of DT required to give a response of 0.4. T' is the concentration of DT required to give the same response in the presence of Con A. The ratio T'/T was calculated for Con A concentrations of 5, 10, 15, 20, and 25 $\mu\text{g/ml}$.

The latter possibility is unlikely because Con A (16), WGA (17), and DT (2) do not appear to be glycoproteins. Furthermore, we were unable to find any evidence for the interaction of DT with agarose-insolubilized Con A.

The Effect of Oligosaccharides on DT. Con A binds to oligosaccharides containing mannose (18), and WGA binds to those containing GlcNAc (19). It is possible that the DT receptor site contains these saccharide units. We attempted to inhibit DT with either 50 mM MeMan or 50 mM GlcNAc or both. GlcNAc had no effect whereas MeMan gave slight inhibition.

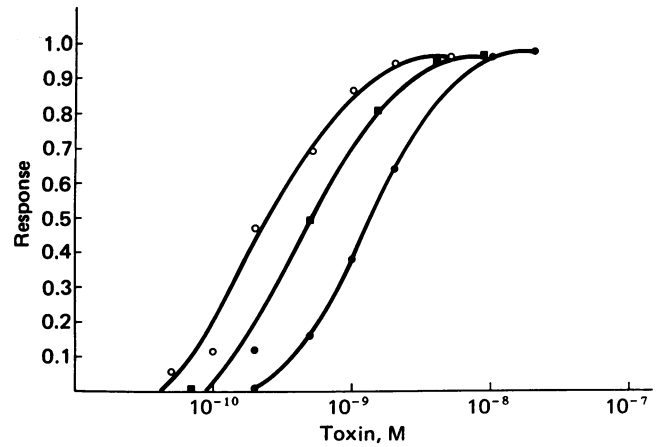


FIG. 5. Effect of ovalbumin glycopeptide and the *Salmonella cholera suis* 5210 mannan on the response of cells to DT. \circ , DT alone; \blacksquare , ovalbumin glycopeptide, 24 $\mu\text{g/ml}$; \bullet , *S. cholera* mannan, 200 $\mu\text{g/ml}$.

Both monosaccharides together gave the same result as MeMan alone. We then tried oligosaccharides containing mannose, GlcNAc, or both.

The structures of the oligosaccharides are shown in Fig. 4. The cell wall polysaccharide of *Salmonella cholera suis* 5210 and the ovalbumin glycopeptide inhibited DT; the others were inactive. The dose-response curves for DT in the presence of the inhibiting polysaccharides are shown in Fig. 5. Once again, the inhibition could be overcome by increasing the DT concentration. The concentration of polysaccharide required to inhibit 1.3 nM DT by 50% was 25 $\mu\text{g/ml}$ for the *Salmonella cholera* polysaccharide and 55 $\mu\text{g/ml}$ for ovalbumin glycopeptide. We also found that whole egg ovalbumin inhibited DT to the extent expected for its carbohydrate content, indicating that preparation of the glycopeptide by Pronase digestion of ovalbumin did not impart an inhibitory property to the gly-

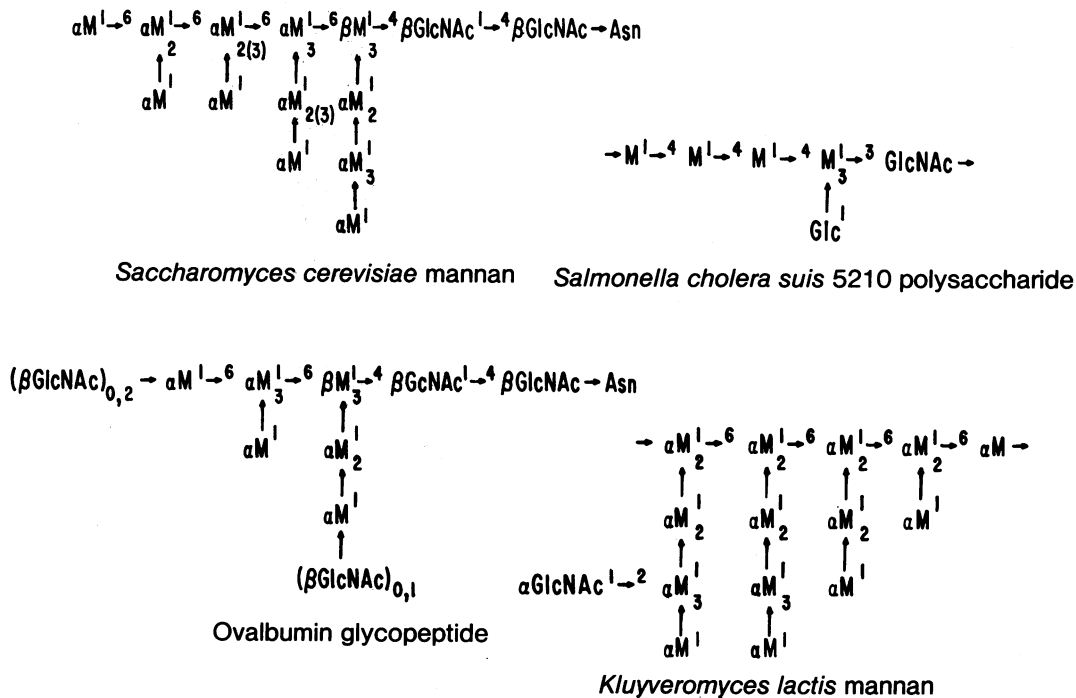


FIG. 4. Structures of oligosaccharides tested for inhibition of DT. M, mannose; GlcNAc, *N*-acetylglucosamine; Glc, glucose; Asn, asparagine. The linkage between sugars is not given where it is not known. The complete structure of the *Salmonella cholera* polysaccharide is unknown. Refs. to these structures are: *S. cerevisiae* (24, 25); *S. cholera* (26, 27); ovalbumin glycopeptide, (11, 24); *K. lactis*. (25).

copeptide. In addition, boiling the oligosaccharides did not affect their ability to antagonize DT. Cells that were treated first with oligosaccharide, washed with medium, and then exposed to DT were still sensitive. These results suggest that the inhibitors are interacting with DT.

DISCUSSION

The lectins Con A, WGA, and Suc-Con A are potent inhibitors of DT. We used a Schild analysis (13–15) to investigate the effect of Con A on the response of cells to DT, and the results indicate that these two proteins compete for a common target. Con A does not protect the cells if DT is added 15 min prior to the lectin; after DT has entered the cells, they cannot be rescued. The cells are protected when Con A is not in the medium, provided that the cells are pretreated with the lectin. These observations suggest that the site of protection is probably associated with the cell surface and that, after some critical event, Con A cannot inhibit the toxin.

The apparent K_D of Con A for the site of competition is 3×10^{-8} M. This is the same order of magnitude as the apparent K_D of DT for its receptor on HeLa cells (12) but indicates a more avid interaction than that generally reported for the binding of Con A to whole cells (20). It is known that mouse and rat cell lines are refractory to DT and probably lack the DT receptor (21), yet these cells bind Con A. It is likely that the target on hamster cells for which Con A and DT compete represents a small high-affinity subset of the total Con A receptors, and this subset is absent from the surface of mouse and rat cells.

Although Suc-Con A is not as effective an inhibitor as Con A, it is still a good inhibitor of the toxin. The ability to inhibit is independent of the valence of Con A and probably is independent of the reorganization of surface receptors attributed to tetravalent Con A (22, 23).

We could not construct a reliable Schild plot for WGA because our preparations of this lectin became insoluble in the medium at $>10 \mu\text{g/ml}$. The shape and maximum of the dose-response curve for DT in the presence of a low concentration of WGA suggested that this lectin is also a competitive inhibitor of the toxin.

Little is understood about the interaction of DT with the cell surface or about the events that lead to translocation of the toxin molecule to the cytoplasm. By direct measurement, Boquet and Pappenheimer (21) obtained evidence that there are only about 4000 receptor sites per HeLa cell. Ittelson and Gill (12) studied a nonlethal mutant DT protein and showed (with a Schild analysis) that this protein was a competitive inhibitor of DT with an apparent K_D of about 10^{-8} M (12). After the reversible reaction of DT with its receptor, there is an irreversible step beyond which the cells are committed to intoxication. A reasonable explanation for our lectin data is that these carbohydrate-binding proteins interact with toxin receptor. This idea suggests that at least part of the information directing DT binding to cells may be an oligosaccharide sequence. We cannot rule out the possibility that the lectins interact at a site distinct from but close enough to the toxin receptor to inhibit sterically the approach of DT or that before the irreversible step there may be one or more reversible steps subsequent to toxin binding which could require participation of a lectin receptor and which could be blocked by a lectin. Direct evidence for the interaction of DT with an oligosaccharide awaits demonstration of a DT-carbohydrate complex. However, one prediction of the hypothesis that DT interacts with a carbohydrate-containing receptor is that the toxin would be inhibited by certain oligo-

saccharides. This prediction was verified: the cell wall polysaccharide of *Salmonella cholerae suis* and the ovalbumin glycopeptide are inhibitors.

A comparison of the oligosaccharide structures in Fig. 4 does not allow an accurate assignment of the parts of these molecules that are important for inhibition, but several suggestions can be made. The major difference between the yeast mannan and the ovalbumin glycopeptide is the variable presence on the latter of terminal GlcNAc in β linkage to mannose (11, 24, 25). Because the yeast mannan is not an inhibitor, the terminal GlcNAc residues seem to be important. Second, the *Salmonella cholerae* polysaccharide is an inhibitor but the published structure of the repeating unit does not contain a terminal GlcNAc (26, 27). However, this does not exclude the possibility that terminal GlcNAc residues are present in this polysaccharide. Third, the *Kluyveromyces lactis* mannan contains a terminal GlcNAc linked α -(1–2) to mannose and this oligosaccharide is not an inhibitor. This suggests that, if a terminal GlcNAc is important for inhibition of the toxin, then the configuration of the linkage connecting it to the rest of the molecule is also important. Fourth, GlcNAc does not inhibit DT, and MeMan is at best a poor inhibitor. This need not be inconsistent with the other data. Kornfeld *et al.* (28) showed that *Lens culinaris* lectin binds avidly to polysaccharides containing mannose and GlcNAc but the monosaccharides barely inhibit this reaction. The information for *Lens culinaris* lectin binding resides in the sequence of the sugars and this may also be true for DT. Finally, the relatively high concentrations of polysaccharides required to inhibit DT suggest that there is probably only a slight homology between the inhibitors and the true toxin receptor.

In recent years, it has become clear that the mechanisms of action of some protein hormones and plant and bacterial toxins have much in common (6, 21, 29). One generalization that has emerged is that the initial interaction of some of these proteins with cells is a result of their lectin-like carbohydrate binding properties. Our results suggest that DT fits this pattern.

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