

# Diphtheria toxin forms transmembrane channels in planar lipid bilayers

(membrane protein/voltage-dependent conductance/protein integration into membranes/lysosome/endocytosis)

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**ABSTRACT** When exposed to a lipid bilayer, diphtheria toxin binds to it and forms transmembrane, voltage-dependent, anion-selective channels. The mean ( $\pm$ SD) conductance of these channels in asolectin membranes is  $6.2 \pm 0.7$  pmho ( $\mu$ S) in 0.2 M NaCl and  $20 \pm 2$  pmho in 1.0 M NaCl. The rate of channel formation depends on the pH in the toxin-containing compartment; it is very low at pH  $>5.0$  and increases abruptly as the pH decreases from 4.9 to 4.0. Binding of toxin to the membrane is also pH dependent, being unmeasurable at pH 7 and increasing monotonically with decreasing pH. The rate of channel formation depends upon membrane potential as well; channels form only at negative potentials. These channels are permanent in the time scale of the experiments (about 1 hr). The membrane conductance caused by the channels is also voltage dependent, being constant at positive potentials and decreasing at negative potentials. Hence, the current-voltage curve is linear at positive potentials and sublinear at negative potentials. The conditions necessary for insertion of toxin into the bilayer and formation of channels are similar to those that prevail inside the lysosome. Thus, these results lend credence to the idea that toxin enters the cytoplasm from the lysosomal compartment.

The amino-terminal portion of diphtheria toxin (DT) (fragment A;  $M_r$  22,000) arrests protein synthesis in most mammalian cells by catalytically inactivating elongation factor 2 (1). In order to interact with elongation factor 2, fragment A must first cross a membrane barrier and enter the cytoplasm (2, 3). The first step in this process occurs when the toxin associates with a cell surface receptor (2). The subsequent events by which fragment A is translocated into the cytoplasm are unknown.

Boquet *et al.* (4, 5) have suggested a mechanism for translocation in which the toxin forms a transmembrane channel in the plasma membrane through which fragment A passes into the cytoplasm. This suggestion is made plausible by the fact that several microbial toxins have been shown to form ion-conducting channels in model lipid membranes. Tosteson and Tosteson (6) showed that cholera toxin forms channels in planar lipid bilayers that contain the ganglioside  $G_{M1}$ . Schein *et al.* (7) showed that colicin K forms channels in planar lipid bilayers, and Kayalar and Luria (8) and Tokuda and Konisky (9) showed that this and several other colicins increase the permeability of lipid vesicles (liposomes). Furthermore, Kagan and Finkelstein (10) reported that a DT fragment, crossreacting material 45 (CRM45), forms channels in lipid bilayers. CRM45, a toxin fragment of  $M_r$  45,000, lacks the carboxyl-terminal portion of the toxin and is produced by *Corynebacterium diphtheriae* that are lysogenic for the mutated phage  $\lambda 45$  (2). CRM45 has an accessible hydrophobic domain (4) and thus it binds to liposomes (5). However, CRM45 is much less toxic to cells than is whole diphtheria toxin (2). Thus, it remains to be shown that intact toxin itself can form transmembrane channels.

It has also been suggested that fragment A may enter the cy-

toplasm after the DT molecule is taken up by endocytosis (11). Fragment A then escapes from the endocytotic or the lysosomal compartments either by crossing the lysosomal membrane (perhaps by a variant of the channel model) or by rupture of the vesicle. The evidence that lysosomes are involved in intoxication includes the fact that cells are protected from intoxication when endocytosis is inhibited by cooling the cells (12) or by treatment with lysosomotropic drugs such as ammonium ion (13) and chloroquine (2). These drugs, all weak bases, share the property that they accumulate in the acidic environment of the lysosome and increase the lysosomal pH. Apparently, the acidic pH of the lysosome may facilitate intoxication.

Recent work by Draper and Simon (12) and by Olsnes (14) indicates that mimicking the lysosomal environment at the plasma membrane (by lowering the pH) enables toxin to attack a cell even when endocytosis is stopped. Furthermore, Alving *et al.* (15) have shown that the binding of DT to liposomes is a function of pH. These results prompted us to study the effect of DT on planar lipid bilayer membranes to determine if DT does indeed form transmembrane channels, to elucidate the conditions required for channel formation, and, thus, to gain insight into the molecular mechanisms of intoxication.

## MATERIALS AND METHODS

DT was obtained from Connaught Laboratories (Willowdale, ON); after purification by DE-52 chromatography it showed a single band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (16). Toxoid was prepared by incubating DT in 2% formaldehyde for 48 hr followed by dialysis against phosphate-buffered saline for 24 hr. Abrin (17) and fragment A (16) were prepared according to published procedures.

Phosphatidylethanolamine (PtdEtn; Sigma) and asolectin (soybean phospholipids, L- $\alpha$ -lecithin; Sigma) were partially purified by the method of Kagawa and Racker (18) and stored as hexane solutions at  $-80^\circ\text{C}$  until use. Tocopherol was added to asolectin as an antioxidant to a concentration of 2% (wt/wt). Solutions were buffered with 10 mM phosphate (pH 7.0 or 6.0) or 10 mM acetate (pH 5.0, 4.85, or 4.0).

Planar lipid bilayer membranes were made by the apposition of two lipid monolayers as described (19). Two adjacent compartments in a Teflon chamber were separated by a thin (12.5  $\mu\text{m}$ ) Teflon partition (Yellow Springs Instruments) in which a small (typically, 250  $\mu\text{m}$ ) aperture had been punched. The aperture was treated with a 1% solution of squalene (Sigma) in *n*-pentane. Then, buffer solution was introduced into the compartments such that it was still below the level of the aperture. A phospholipid monolayer was formed by spreading 10  $\mu\text{l}$  of a

Abbreviations: DT, diphtheria toxin; CRM45, cross-reacting material 45; PtdEtn, phosphatidylethanolamine.

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0.5% solution of phospholipid in pentane on the buffer in each compartment and waiting for the solvent to evaporate. Thereafter, buffer solution was injected under the monolayers to raise them over the aperture, thus forming a bilayer. Bilayer formation was continuously monitored by the increase in electrical capacitance between the two compartments (19). All bilayers were formed and studied in 0.2 M NaCl/10 mM acetate, pH 4.85, unless otherwise noted. All experiments were performed at room temperature.

The compartment to which DT was added was continuously stirred. This compartment is defined as being at ground potential, and positive current is defined as positive ions moving from the DT-free compartment to the DT-containing compartment. Current was measured by using Ag/AgCl electrodes and was converted to voltage by using an AD515 operational amplifier (Analog Devices, Norwood, MA) with a feedback resistor of 1 G $\Omega$  (response time, 500  $\mu$ s). The signal was recorded on a Racal 4DS fm tape recorder (Racal, Rockville, MD). Voltage was monitored with Corning calomel electrodes and a Keithley 600B electrometer.

The binding experiments were performed as follows. A membrane was formed at a given pH and a positive potential was applied to the membrane. DT was then added and the system was incubated for 15 min. Then, the DT-containing compartment was perfused with saline at the same pH. The washing was done with a volume 5 times the capacity of the compartment (6 ml vs. 1.2 ml). Then the pH of the DT-containing chamber was adjusted to 4.85. Thereafter, the potential was switched to  $-40$  mV, and the current through the membrane 4 min later was taken as a measure of the number of toxin molecules bound to the membrane.

## RESULTS AND DISCUSSION

**DT Increases Membrane Conductance.** The conductance of unmodified lipid bilayer membranes is very small, generally between 1 and 5 pmho ( $\mu$ S) in 0.2 M NaCl (10 nmho/cm<sup>2</sup>), allowing very small changes in membrane conductance to be measured readily. The magnitude of this small conductance is illustrated in Fig. 1 by the small current that passed through the membrane when a potential of  $-40$  mV was applied. When DT was added to the solution bathing the membrane, the membrane conductance increased dramatically. After addition of DT to the solution to a concentration of 0.75 nM the conductance increased after a lag time of about 1–2 min. Such a lag can be accounted for by the finite rate of diffusion of DT from the bulk aqueous phase to the surface of the membrane. After the lag time, the conductance started increasing in discrete steps until, after three or four overlapping transitions, discrete increases were not discernible. Note, however, that the conductance record is noisy.

**DT Forms Transmembrane Channels.** The behavior of the toxin-induced increase in membrane conductance suggests that the toxin forms discrete ion-conducting channels in the membrane and that each conductance step corresponds to the opening of a single channel. In order to resolve individual conductance steps, DT at only 30 pM was added to the aqueous phase, and the aqueous salt concentration was increased to 1 M NaCl, in order to increase the amplitude of the steps. The first transition in the current trace (Fig. 2) is due to the switch in potential from 0 to  $-60$  mV. The spike is due to the transient capacitive current and the conductance of the membrane is about 25 pmho. After 10 sec, the current abruptly increased by 1.2 pA to a more negative value. This current step was followed by others, all of the same size. The size of these current steps corresponds to a mean ( $\pm$ SD) conductance change of  $20 \pm 2$  pmho.

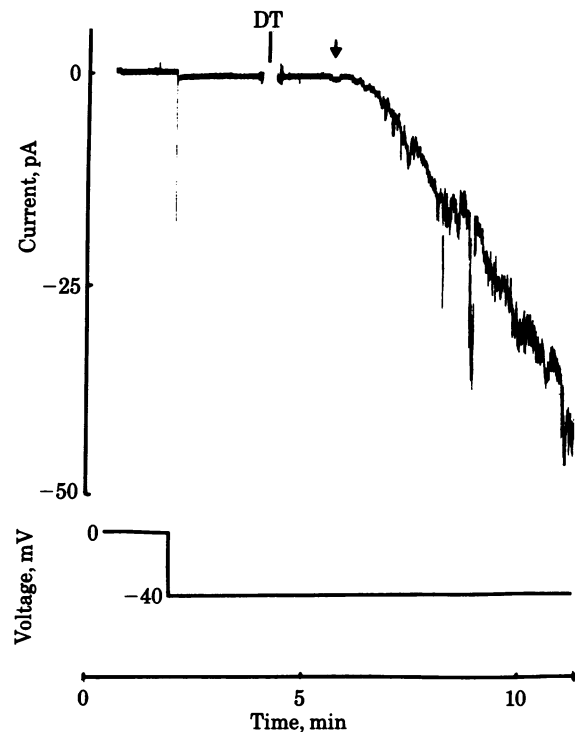


FIG. 1. Development of toxin-induced conductance in symmetric planar lipid bilayers. A voltage step of  $-40$  mV was applied across the membrane and held constant throughout the record, as shown in the lower trace. The current flowing through the bilayer in response to the applied voltage is shown in the upper trace. DT was added to one compartment to a concentration of 750 pM (50 ng/ml). The electrical artifact introduced by the addition of DT was deleted from the record as indicated by the interruption in the trace. After a 2-min delay, the current increased (became more negative), indicating an increase in conductance. Note the opening and closing of a single toxin channel as indicated by the arrow. The bathing solution was 0.2 M NaCl at pH 4.85.

In 0.2 M NaCl, the single-channel conductance was  $6.2 \pm 0.7$  pmho. Occasionally, the current jumped to a lower level (upward step on the record). These steps correspond to one of the channels closing transiently. Thus, individual channels may open or close transiently.

When a negative potential was applied, the membrane conductance continued to increase as long as toxin was present in the aqueous phase. When the toxin-containing chamber was washed free of toxin, the conductance ceased to increase and remained constant with time. This implies that, although they fluctuate between open and closed states, the channels do not disappear completely. Thus, within the time scale of these experiments (about 1 hr), the toxin channels are permanent entities in the membrane.

**Channel Formation Depends upon Voltage and pH.** The membrane potential has a large effect on the rate of development of the conductance. In the presence of DT, when the membrane potential was held at a positive value, no channels formed even after 30 min of observation. When a moderate negative potential was applied—for example,  $-20$  mV—channels did form, but slowly. As the membrane potential was made more negative, the rate of channel formation increased.

The pH of the solution in the toxin-containing chamber also has a large effect on the rate of development of the conductance (Fig. 3). Fig. 3 *Inset* shows the current through three membranes held at the same potential and treated with the same concentration of DT, 750 pM, but at different pHs. The rate of de-

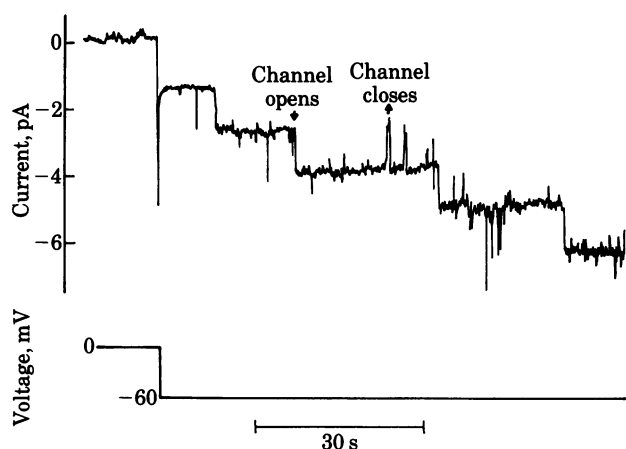


FIG. 2. Single channels of DT. DT was added to 30 pM (2 ng/ml) and a potential of  $-60$  mV was applied. The initial jump in the record is due to the application of the potential; note the capacitive transient. Thereafter, the current increased (became more negative) in steps of 1.2 pA corresponding to conductance changes of 20 pmho. The bathing solution was 1.0 M NaCl at pH 4.85.

velopment of the conductance was greatest at pH 4, less at pH 5, and much less at pH 6. It is clear that an acidic pH is required for DT to form channels rapidly in lipid bilayers.

**DT Binding Is Also pH Dependent.** Conceptually, channel formation in this system can be considered to consist of two steps (i) binding of the toxin to the membrane and (ii) opening of the channel:



These two steps may be concerted or they may be distinct. The

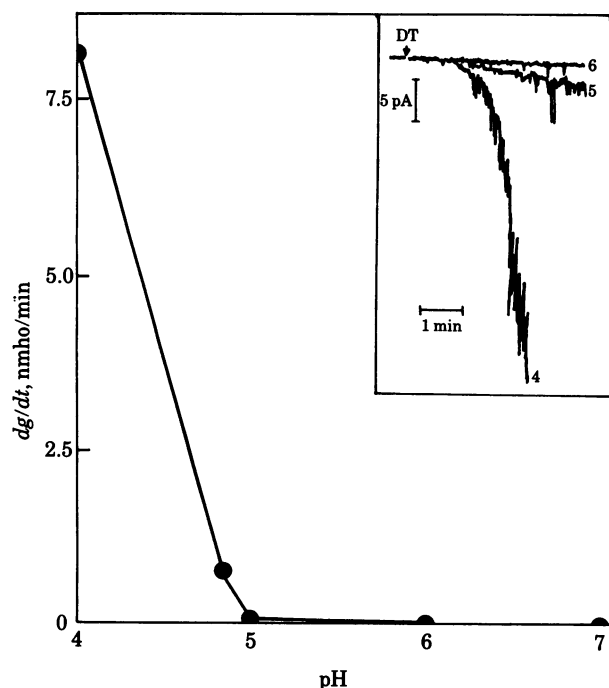


FIG. 3. Effect of pH on the rate of conductance development ( $dg/dt$ ). DT was added, to a concentration of 1.5 nM (100 ng/ml), to the solution on one side of a lipid bilayer membrane held at  $-40$  mV. Examples of the resulting membrane currents are shown in the *Inset* for pH 4.0, 5.0, and 6.0.

effect of membrane potential on channel formation allowed us to determine that these steps are distinct. When the membrane was held at  $+40$  mV and DT was added, no channels formed. At this point, either the toxin was not bound to the membrane, or it was bound but was not capable of channel formation. When the toxin was then washed out of the chamber, and the potential was switched to  $-40$  mV, channels were observed to form. These results suggest that the toxin was bound to the membrane at  $+40$  mV but was prevented from forming a channel by the positive potential. Thus, binding and channel formation are distinct steps.

We studied the rate of this binding at different pH values by adding DT at a given pH with a positive applied potential and incubating for 15 min to give the toxin time to bind to the bilayer. Then, the chamber was washed free of toxin, the pH was adjusted to 4.85, and the potential was switched to  $-40$  mV. The increase in conductance after 4 min was taken as a measure of toxin binding. The results of this type of experiment are shown in Fig. 4. It is apparent that, in *asolectin* membranes, toxin binds most rapidly at low pH.

Comparison of Figs. 3 and 4 shows that the rate of toxin binding is significant at pH 6, but the rate of channel formation is not. Bound molecules do not form channels at a measurable rate at pH 6, suggesting that the channel formation step ( $DT_{\text{bound}} \rightleftharpoons DT_{\text{conducting}}$ ) is also pH dependent.

**Properties of the Toxin-Induced Conductance.** The membrane potential not only affects the rate of channel formation but also affects channels in a completely different way after they are formed. Fig. 5 is a current trace from a membrane that first was treated with DT at a negative potential so that channels formed, after which the chamber was washed free of toxin so that the conductance reached a steady value. When the potential was switched to  $+40$  mV, the current instantaneously jumped to a positive value and then relaxed in a finite time to a higher level. When the potential was switched back to its original negative value, the current jumped instantaneously and then relaxed to its lower, original value. Finally, the current became zero when the potential was stepped to zero. By measuring the steady-state conductance as a function of potential, we found that the conductance is constant at positive potentials and decreases at negative potentials, suggesting that the probability for the channel

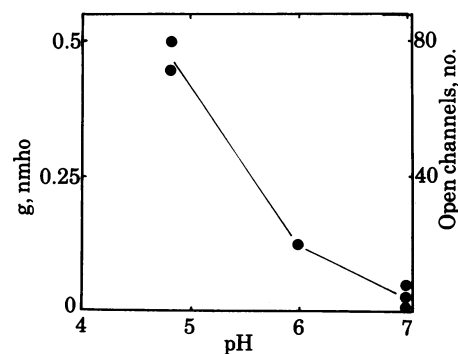


FIG. 4. Conductance due to membrane-bound toxin. DT was added to the solution on one side of a lipid bilayer to a concentration of 1.5 nM (100 ng/ml) at a given pH. The membrane potential was held at  $+20$  mV for 15 min and then the DT-containing chamber was washed free of toxin. The solution in the washed chamber was adjusted to pH = 4.85, and the potential was switched to  $-40$  mV. The resulting membrane conductance is plotted as a function of the initial pH. This conductance is due to membrane-bound toxin, and its magnitude is taken as a measure of the amount of toxin bound with only 15 min of incubation. The binding has not saturated; hence, this procedure measures the rate of toxin binding.

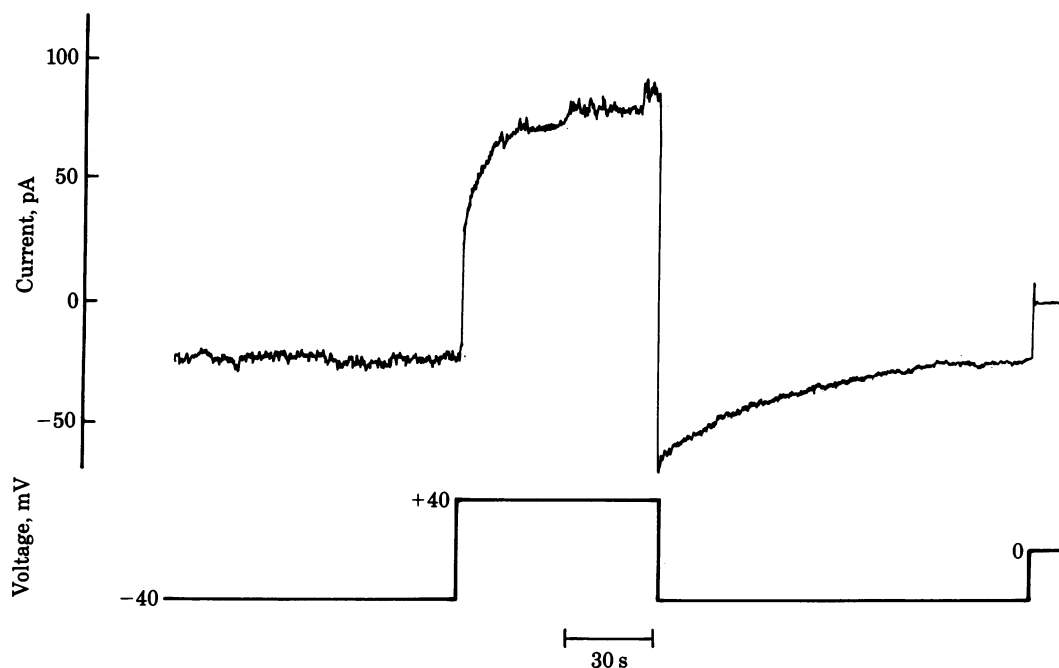


FIG. 5. Voltage-dependence of the toxin-induced membrane conductance. DT was added to the solution on one side of the membrane to a concentration of 750 pM (50 ng/ml). The potential was held at  $-40$  mV, and after 15 min the chamber was washed free of toxin. Then, the potential was switched to  $+40$  mV and back to  $-40$  mV. Note the increase in current at positive potentials and the decrease at negative potentials. The bathing solution was 0.2 M NaCl at pH 4.85.

to be in a conducting state is greater at positive potentials. Thus, the membrane acts as a rectifier, resulting in an asymmetric current-voltage curve which is linear (ohmic) at positive potentials and sublinear at negative potentials. The asymmetry of the current as a function of voltage indicates that there is a vectorial insertion of the DT molecule into the bilayer; a random insertion would imply no preferred orientation and hence would yield a symmetric current-voltage curve. This suggests a specific interaction of the toxin molecule with the membrane. This specific, vectorial insertion of the toxin molecule may also have some function in the intoxication process.

In order to characterize the channel further, we studied the selectivity of the toxin-induced conductance. It was necessary to use membranes of different composition than those used in the above experiments because the significant negative surface potential of asolectin membranes influences the ion selectivity by increasing the concentration of cations at the membrane surface. We formed membranes from PtdEtn which have only a small surface charge (20). The characteristics of the toxin-induced conductance in PtdEtn membranes were similar to those for membranes made from asolectin, except that the conductance was smaller; more DT was needed to achieve the same level of conductance. This agrees with the results of Alving *et al.* (15) that toxin binds more strongly to negatively charged liposomes.

The open circuit potential difference of bilayers separating two compartments with different NaCl activities showed that the conductance is anion selective with a selectivity ratio  $\text{Cl}^-:\text{Na}^+$  of 6:1.

In order to start dissecting the molecular mechanism by which toxin forms ionic channels, we measured the rate of channel formation as a function of DT concentration. We found that the rate of conductance increase is proportional to the square of the toxin concentration, suggesting that the channels are formed from dimers of toxin. These aggregates may be preformed in so-

lution or they may form after the binding of monomers to the membrane.

**Toxin Analogues.** It was necessary to test if this increase in membrane conductance due to DT was the result of a nonspecific interaction of protein with the membrane or of specific interaction related to the structure of DT. This was done by treating the membrane with other toxins and with toxin analogues including abrin (a plant toxin), fragment A, and toxoid (an inactive form of toxin). Toxoid had no effect on membrane conductance, even at a concentration of 15 nM (Fig. 6). DT, on the other hand, had a large effect at 0.15 nM. Fragment A and abrin also had no effect on membrane conductance under the same conditions. Thus, the observed increase in conductance is specific for DT.

**Insights into the Mechanism of DT Intoxication.** The results presented here are relevant to the mechanism by which fragment A of DT may enter the cell cytoplasm. We have demonstrated that DT inserts into a lipid bilayer at low pH. This would likely occur in the living cell wherever the toxin was placed in an acidic environment. Such an environment exists in the lysosomes, and there is evidence that the toxin enters the lysosomes after adsorptive endocytosis (21). It is also possible that the toxin could encounter acidic conditions elsewhere—for example, within endocytotic vesicles. We are aware, however, of no information on the pH within these vesicles. Appropriate conditions similar to those needed for channel formation in the bilayer could also occur anywhere on a membrane where there was a high local concentration of protons.

We do not know if fragment A of the toxin is translocated through the membrane when the toxin inserts. However, it has been suggested (4, 5) that an expected prerequisite for the translocation of fragment A through an intact membrane is conversion of the toxin into a membrane protein.

The fact that the toxin molecule becomes a membrane protein and forms a channel may have a direct significance for the

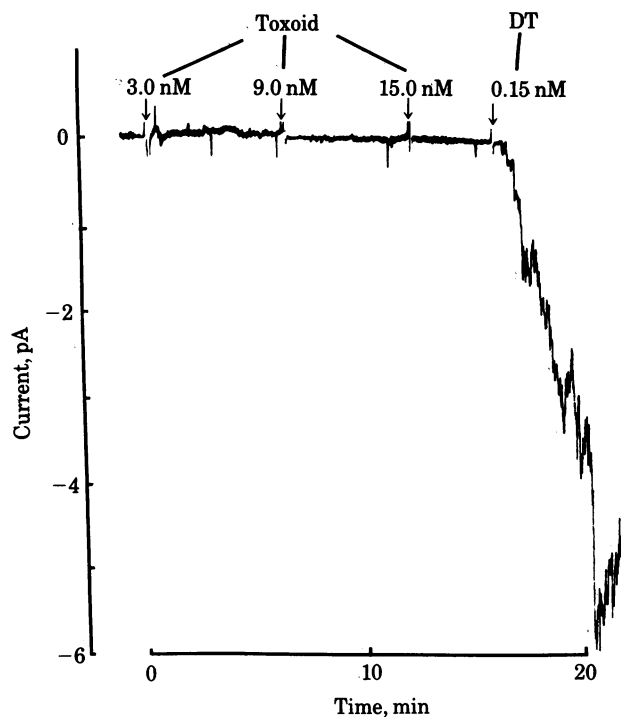


FIG. 6. Comparison of toxoid and DT. Toxoid was added to the solution on one side of the membrane in several increments to a final concentration of 15 nM (1  $\mu$ g/ml) with the membrane held at  $-40$  mV. Thereafter, DT was added to a concentration of 0.15 nM (10 ng/ $\mu$ l). Only DT changed the membrane conductance.

intoxication process. For example, fragment A may be transported across the membrane barrier through the toxin channel as suggested by Boquet *et al.* (4, 5). However, the low channel conductance implies that the diameter of the channel is quite small ( $\approx 5$  Å), rendering this possibility unlikely. Alternatively, the channel properties of the toxin molecule might not be directly involved in intoxication. Instead, incorporation of the protein into the hydrophobic region of the membrane may result in a conformational change which translocates fragment A across the membrane, exposing the enzymatic moiety. The channel may thus be a consequence of the insertion of the toxin molecule into the bilayer. In either case, the interaction of the toxin molecule with the hydrophobic interior of the membrane may be a crucial step in the mechanism of DT intoxication.

In light of this, we suggest that the approach we have taken to assess the mechanism of DT interaction with lipid bilayers may be a means to study the mechanism of protein integration into bilayers and may provide insights into the specific structural requirements for membrane protein assembly. Such structural requirements have been invoked by Blobel (22) and by Wickner

(23) to explain the insertion of proteins into biological membranes.

## CONCLUSIONS

DT forms channels in lipid bilayer membranes. These channels are voltage dependent and anion-selective and have a single-channel conductance of 6.2 pmho in asolectin membranes in 0.2 M NaCl. The rate at which these channels form depends upon pH and upon membrane potential, such that the rate increases at acidic pH and at negative potentials. Potential has two distinct effects on the channels. A negative potential is needed to induce the channels to form. Once formed, however, the channels tend to be in a low conductance state at negative potentials and in a high conductance state at positive potentials. The nonlinear dependence of the rate of channel formation on DT concentration suggests that these channels are formed from aggregates of the toxin molecule.

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