Diphtheria Toxin Entry into Cells is Facilitated by Low pH

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ABSTRACT At neutral pH, NH_4Cl and chloroquine protected cells against diphtheria toxin. A brief exposure of the cells to low pH (4.5–5.5) at 37°C completely abolished this protection. When, to cells preincubated with diphtheria toxin and NH_4Cl, neutralizing amounts of anti-diphtheria toxin were added before the pH was lowered, the toxic effect was considerably reduced, but it was not completely abolished. A much stronger toxic effect was seen when antibodies were added immediately after incubation at low pH.

Upon a short incubation with diphtheria toxin at low pH, the rate of protein synthesis in the cells decreased much faster than when the normal pH was maintained. The data suggest that, at low pH, diphtheria toxin (or its A fragment) penetrates directly through the surface membrane of the cell. The possibility is discussed that, when the medium has a neutral pH, the entry of diphtheria toxin involves adsorptive endocytosis and reduction of the pH in the vesicles possibly by fusion with lysosomes. Low pH did not facilitate the entry of the closely related toxins abrin, ricin, and modeccin.

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It is well established that the cytotoxic action of diphtheria toxin can be inhibited by ammonium chloride (2, 7, 9, 10) and by chloroquine (12, 17). These compounds also protect against the toxic lectin modeccin, albeit to a lesser extent (17). Both diphtheria toxin (for review, see reference 16) and modeccin (15) consist of two functionally different moieties, the B fragment or B chain, respectively, which bind to cell surface receptors, and the A fragment or A chain which enter the cytoplasm and inactivate components of the protein synthesis machinery. Because ammonium chloride and chloroquine apparently inhibit neither the binding of the toxins to the cells nor the action of the toxin A moieties in cell-free systems (2, 10, 17), they may inhibit the transport of the toxins from the cell surface into the cytoplasm.

Considerable amounts of diphtheria toxin and modeccin can be taken into cells by adsorptive endocytosis, but it has not been known whether this mechanism is necessary for the intoxication process. In the case of diphtheria toxin, its rapid degradation to amino acids indicates that most of the internalized toxin is transferred to lysosomes (5). When ammonium ions or chloroquine are present in the medium, ammonia and uncharged chloroquine diffuse into the cells. In the acidic interior of the lysosomes, they become protonated and thus increase the pH in the lysosomes (22). If therefore the toxin requires low pH to penetrate the cell membrane, the protective effect of the two compounds would easily be explained. Recently, Helenius et al. (6) showed that Semliki Forest virus was able to fuse with membranes only at the low pH present in lysosomes and that addition of ammonium ions and chloroquine to the medium prevented virus entry. To investigate the possibility that low pH is also necessary for the transfer of the toxins (or their A chains) to the cytoplasm, we have tried to circumvent the protection afforded by ammonium chloride and chloroquine by incubating toxin-treated cells at a low pH for a short time and we have tested the effect of low pH on the rate of toxin entry in the absence of protective agents.

MATERIALS AND METHODS

Toxins and Antitoxins

Modeccin was extracted from roots of *Adenia digitata* and purified as described earlier (14). Diphtheria toxin was obtained from Connaught Laboratories (Willowdale, Ontario, Canada) and crm 45 was a generous gift from Professor A. M. Pappenheimer, Jr. (Harvard University). Horse antiserum to diphtheria toxin was obtained from The National Institute for Public Health (Oslo, Norway).

Cells

African green monkey kidney cells (Vero) were obtained from The National Institute for Public Health (Oslo). The cells were maintained as monolayer cultures in medium 199 with Earle's Modified salts (Gibco, Glasgow, Scotland) and 10% fetal calf serum.

Measurement of Cytotoxicity

Cells were transferred to disposable trays with 24 wells, 2 cm^2 each (Costar, Cambridge, Mass.) (5×10^4 cells in 1 ml per well), the day before the experiment. Toxin, ammonium chloride, and neutralizing amounts of antitoxin were added as indicated in legends to figures. In some experiments the cells were incubated at different pH values which were obtained by adding sufficient H_3PO_4 to a buffer containing 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 3.2 mM Na_2HPO_4, and 137 mM NaCl. Protein synthesis was measured at the end of each experiment by washing the cells with the buffer (pH 7.2) and then incubating them for 30 min with Eagle's minimum essential medium containing 21 mM HEPES (pH 7.7) instead of bicarbonate, no unlabeled leucine, and 25 nCi [^{14}C]leucine. The medium was then removed, the cells were dissolved in 0.1

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M KOH, trichloroacetic acid was added to a final concentration of 10% (wt/vol), and the acid-precipitable radioactivity was measured.

RESULTS

Inability of Ammonium Chloride and Chloroquine to Protect Cells against Diphtheria Toxin at Low pH

When 10 mM NH_4Cl was added to Vero cells in medium at pH 7.2, the cells were fully protected even against very high concentrations of diphtheria toxin (Fig. 1). However, the protective effect of ammonium chloride was abolished if the cells were exposed for a short time to a pH of 5.5 or lower. In the presence of NH_4Cl, the effect of diphtheria toxin gradually increased as the pH decreased from 7.2 to 5.5. At any pH between 5.5 and 4.0, the toxin was as effective as toxin added to cells at pH 7.2 in the absence of NH_4Cl. It should be noted that in the absence of toxin the ability of cells to incorporate

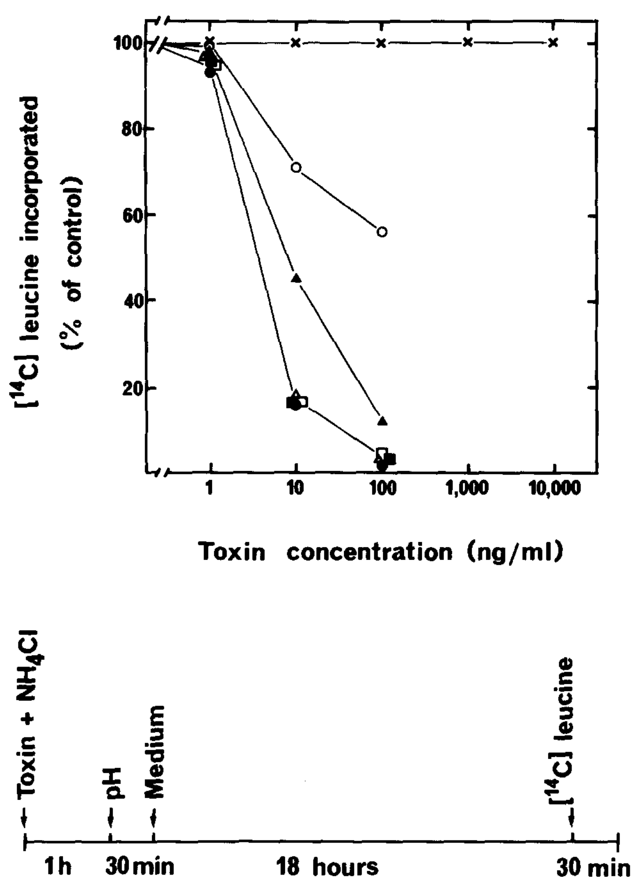

FIGURE 1 Effect of pH on the ability of ammonium chloride to protect cells against diphtheria toxin. Increasing amounts of toxin and 10 mM NH_4Cl were added to cells growing in 24-well disposable trays and the cells were incubated for 1 h at 37°C. Then the medium (and unbound toxin) was removed, phosphate buffers with varying pHs (see Materials and Methods) and 10 mM NH_4Cl were added, and the cells were further incubated at 37°C for 30 min. Then the buffer was removed and growth medium containing 10 mM NH_4Cl was added. After incubation overnight at 37°C the residual rate of protein synthesis was measured as described in Materials and Methods. The results are expressed as percent of the control values (no toxin added) which were ~10,000 cpm. (×) pH 7.2; (○) pH 6.5; (▲) pH 6.0; (△) pH 5.5; (●) pH 4.5; (□) pH 4.0. In one case (■) NH_4Cl was omitted throughout the experiment. The 30-min incubation was in this case at pH 7.2.

[^{14}C]leucine was approximately the same whether or not the cells had been previously exposed to pHs down to 4.5. However, cells exposed to pH 4.0 for 30 min did not fully recover. To achieve the full cytotoxic effect, only a very short incubation at low pH was required. Thus, with cells preincubated with toxin and NH_4Cl for 1 h at 37°C, incubation at pH 4.5 for only 20 s was sufficient to reduce protein synthesis in the cells to approximately the same extent as if NH_4Cl was not present (data not shown). After the addition of NH_4Cl and toxin there appears to be no critical time when the exposure to low pH must be carried out. Thus, the toxic effect developed equally well whether the incubation at low pH was carried out 20 min or 4 h after addition of toxin and NH_4Cl to the cells. Exposure of cells to low pH before toxin was added did not abolish the subsequent protection with NH_4Cl.

The sensitizing effect of the treatment at low pH was strongly temperature-dependent and no toxic effect was obtained if the incubation at low pH was carried out at 0°C (data not shown). Essentially the same results as those obtained with 10 mM NH_4Cl were also obtained with 0.1 mM chloroquine. Because chloroquine is toxic to cells, it was present only during the first 90 min and was then replaced by neutralizing amounts of anti-diphtheria toxin as earlier described (17). Experiments with an incomplete diphtheria toxin molecule (crm 45) indicate that the sensitizing effect of low pH also occurs in the absence of binding of the toxin to its receptors. crm 45 has enzymatic activity in cell-free systems. In relation to toxin it has little effect on intact cells because the lack of the C-terminus renders it unable to bind to the receptors on the cell surface (20). However, when added to cells in concentrations ~1,000 times higher than those required for the whole toxin, crm 45 is able to intoxicate cells. This effect is prevented in the presence of NH_4Cl (3). We found that even with this defective toxin the protective effect of NH_4Cl was circumvented by lowering the pH for 30 min (data not demonstrated). On the basis of this experiment we had expected that mouse cells, which are resistant to diphtheria toxin apparently because of lack of receptors (16), would also become sensitive at low pH. However, in experiments with mouse 3T3 cells, little or no sensitization to diphtheria toxin or to crm 45 was obtained by exposure to low pH.

Low pH Facilitates Entry of Surface-Bound and Endocytosed Toxin

The sensitizing effect of low pH could be caused by (*a*) facilitated penetration of toxin directly through the cell surface membrane, or (*b*) entry into the cytoplasm of toxin present in a compartment not exposed to the cell exterior, e.g., toxin trapped in endocytotic vesicles. To distinguish between these two possibilities, experiments were carried out in which cells were first incubated with NH_4Cl and diphtheria toxin at 37°C for 2 h to allow extensive binding to the cells and uptake of toxin into endocytotic vesicles (5). The cells were then incubated on ice, and anti-diphtheria toxin was added (Fig. 2 and Table I). Provided the normal pH of the medium was maintained, the cells were protected during further incubation at 37°C even after removal of ammonium chloride. This is in agreement with previous authors (2, 10). However, if the cells were incubated at pH 4.5 for 10 min at 37°C after treatment with antitoxin, some toxic effect was seen. It should be noted, however, that, in this case, the toxic effect was less than when antiserum was not added. In contrast, if the pH was reduced

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before antitoxin was added, the toxic effect was almost as strong as without antitoxin (Fig. 2). Clearly, most of the toxic effect induced by low pH is caused by toxin accessible to antibodies, i.e., most likely toxin bound at the cell surface.

In a control experiment in which cells were preincubated with toxin for 2 h at 0°C rather than at 37°C and then treated further as in Fig. 2 *C*, no toxic effect was observed.

Reduction in Lag Time after Exposure of Toxin-treated Cells to Low pH

It has earlier been found that when cells are incubated even with high concentrations of diphtheria toxin, there is time lag before protein synthesis starts to decline (21). This lag time might reflect the time required for toxin to be endocytosed, reach an acidic vesicle, and subsequently enter the cytoplasm. If this were the case, we would expect to dramatically reduce the lag by exposing cells to toxin at a low pH, thereby permitting direct transfer of toxin through the surface membrane. To study this, cells were first exposed to toxin at 0°C for 1 h to allow binding to occur. Unbound toxin was removed by changing the medium, and the cells were incubated briefly in buffer at pH 7.2 or 4.5. We found (Fig. 3 *A*) that the rate of protein synthesis decreased much more rapidly if the cells had been incubated with diphtheria toxin at pH 4.5 than at pH 7.2. At pH 7.2, 100 μg/ml of toxin had the effect of ~0.1 μg/ml at the low pH.

By varying the pH during the pretreatment, we found that there is no critical pH value, but only a gradual increase in the toxic effect as the pH decreases (Fig. 3 *B*).

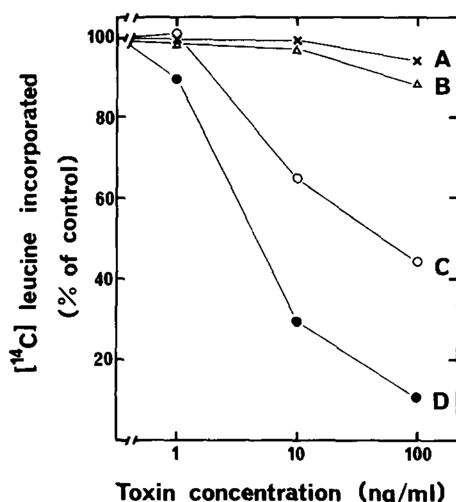

FIGURE 2 Effect of pH on the ability of antibody-sensitive and -insensitive toxin to intoxicate cells. NH_4Cl (10 mM) and various amounts of diphtheria toxin were added to cells growing in 24-well disposable trays. After an incubation of 2 h at 37°C, the trays were cooled on ice and to some of the wells anti-diphtheria toxin was added to neutralize surface-bound toxin (Table I). After 30 min on ice, the medium was removed and 10 mM NH_4Cl in phosphate buffer at pH 7.2 or 4.5 with and without anti-diphtheria toxin was added. After incubation for 10 min at 37°C, the buffer was removed and the cells were incubated in medium containing antitoxin overnight at 37°C. The ability of the cells to incorporate [^{14}C]leucine during 30 min was then measured. (×) Antitoxin added to the cells at 0°C, buffer (pH 7.2) containing antitoxin was used for the 10-min incubation; (○) antitoxin added to the cells at 0°C, buffer (pH 4.5) containing antitoxin was used for the 10-min incubation; (△) antitoxin added after incubation with buffer (pH 7.2); (●) antitoxin added after the incubation with buffer (pH 4.5).

Lack of Effect on Modeccin, Abrin, and Ricin

We have earlier shown that NH_4Cl and chloroquine also protect cells against modeccin (17). Surprisingly, with this toxin the protective effect of ammonium chloride could not be overcome by briefly immersing the cells at pH 4.5 (Fig. 4). The possibility that the persistent protection by NH_4Cl was in this case caused by a rapid dissociation of modeccin from the cells at the low pH was excluded in the following experiment: Cells were first preincubated with modeccin on ice for 1 h to allow maximal binding of the toxin to the cells, then the medium and unbound modeccin were replaced by buffer of pH 4.5 or 7.2. After being incubated at 37°C for 30 min the cells were washed to remove any toxin that had dissociated from the cells. The cells, in fresh medium, were incubated overnight and then their ability to incorporate [^{14}C]leucine was measured. The protein synthesis was inhibited to the same extent under both conditions (data not shown). We also measured the effect of a brief incubation at pH 4.5 on the time lag observed with abrin, ricin, and modeccin under conditions as in Fig. 3 *A*. With these three plant toxins no reduction in the time lag was observed.

DISCUSSION

Our two main findings are (*a*) that a brief reduction in pH increases the rate at which cellular protein synthesis is inhibited by diphtheria toxin, and (*b*) that low pH completely abolishes the protective effect of ammonium chloride. After cells had been exposed to toxin at pH 4.5 for 10 min, their rate of protein synthesis drops as fast as in cells exposed at neutral pH to 1,000 times more toxin.

Ammonia, chloroquine, and related compounds are known to diffuse into the lysosomes and become protonated with a subsequent increase of the pH in the vesicles (13, 18, 19, 22). As first observed by Kim and Groman (9), ammonium chloride in concentrations above 4 mM completely protects cells against diphtheria toxin. Chloroquine in a much lower concentration (0.1 mM) has a similar effect (12, 17). Measurements of intralysosomal pH with a fluorescent probe indicated that 0.1 mM chloroquine and 10 mM NH_4Cl increased the pH approximately the same extent, viz., from the normal value of 4.7–4.8 to ~6.4 (13). This could be sufficient to prevent penetration of the toxin although it should be noted that the data in Fig. 3 *B*

TABLE I

Flow Diagram of Medium Changes and Additions in Fig. 2.

Sample	120 min, 37°C	30 min, 0°C	10 min, 37°C	20 h, 37°C	2 min, 37°C	30 min, 37°C
A	Toxin + NH_4Cl	Antitoxin	NH_4Cl + antitoxin, pH 7.2	Medium + antitoxin	Wash	[^{14}C]leucine
B	Toxin + NH_4Cl	None	NH_4Cl, pH 7.2	Medium + antitoxin	Wash	[^{14}C]leucine
C	Toxin + NH_4Cl	Antitoxin	NH_4Cl + antitoxin, pH 4.5	Medium + antitoxin	Wash	[^{14}C]leucine
D	Toxin + NH_4Cl	None	NH_4Cl, pH 4.5	Medium + antitoxin	Wash	[^{14}C]leucine

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indicate that even at pH 6.5 in the medium the rate of toxin entry is increased.

It was shown first by Kim and Groman (10) that the protective effect of ammonium chloride is reduced by lowering the pH of the medium to 6.3. At this pH the concentration of free ammonia in the medium is very low, and the authors assumed that the protected state was therefore not established. This explanation seems unlikely in our case when a very brief incubation at low pH was sufficient to sensitize the cells even when the protective state had previously been established. Our data indicate that at low pH the toxin mainly penetrates directly through the plasma membrane. We found, however, that even after neutralization of surface-bound toxin by antitoxin, exposure to low pH induced a certain toxic effect. Most likely, this is caused by toxin present in intracellular vesicles.

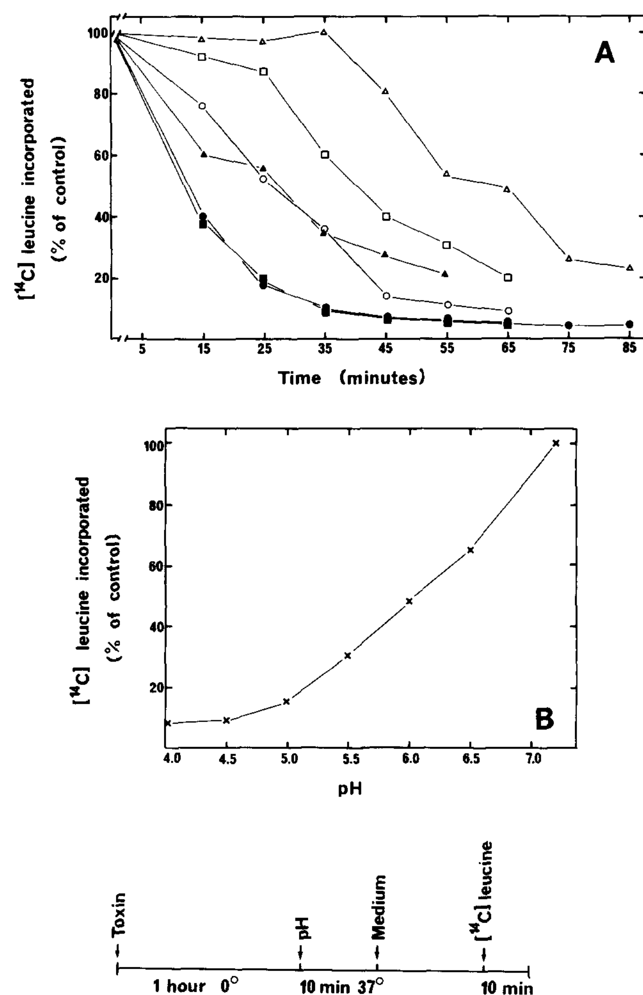

FIGURE 3 Effect of pH on the rate of protein synthesis inhibition after exposure of cells to diphtheria toxin. Diphtheria toxin was added to cells in 24-well disposable trays. After incubation for 1 h at 0°C to allow maximal toxin binding, the medium (and unbound toxin) was removed and phosphate buffer with the indicated pH was added (time zero). After 10 min at 37°C the buffer was removed, and medium prewarmed to 37°C was added. After various periods of time, as indicated on the abscissa in *A*, or after 30 min (*B*), the ability of the cells to incorporate [^{14}C]leucine into acid-precipitable material during a 10-min period was measured. The time points plotted in *A* represent the time from addition of buffer with different pHs to 5 min after addition of [^{14}C]leucine. At each time point [^{14}C]leucine incorporation was measured in control cells treated identically but without toxin. Incorporation in the toxin-treated cells is expressed as percent of this control value (~2,000 cpm). In *A* the different symbols represent: (△) 1 μg/ml toxin, buffer with pH 7.2; (□) 10 μg/ml toxin, buffer wih pH 7.2; (○) 100 μg/ml toxin, buffer pH 7.2; (▲) 0.1 μg/ml toxin, buffer pH 4.5; (●) 1 μg/ml toxin, buffer, pH 4.5; (■) 10 μg/ml toxin, buffer, pH 4.5. In *B* the toxin concentration was 1 μg/ml.

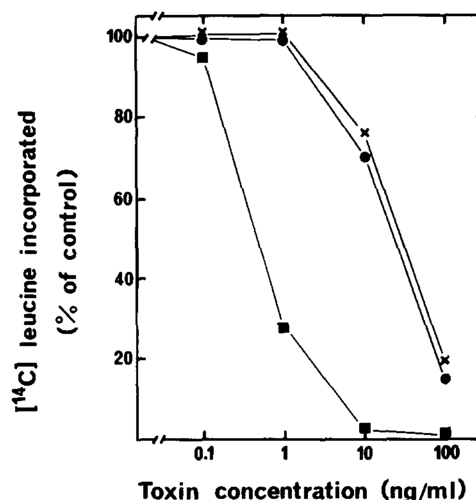

FIGURE 4 Protection of cells against modeccin by ammonium chloride. Increasing amounts of modeccin and 10 mM NH_4Cl were added to cells growing in 24-well disposable trays and the cells were incubated for 1 h at 37°C. Then the medium (and unbound toxin) were removed, phosphate buffers of pH 4.5 and 7.2 as indicated and containing 10 mM NH_4Cl were added, and the cells were further incubated at 37°C for 30 min as in Fig. 1. Subsequently, the buffer was removed, growth medium containing 10 mM NH_4Cl was added, and the cells were incubated overnight at 37°C. Finally, the residual rate of protein synthesis was measured. (X) pH 7.2; (●) pH 4.5. In one case (■) NH_4Cl was omitted throughout the experiment. The 30-min incubation was in this case at pH 7.2.

If it is indeed so that diphtheria toxin can pass rapidly through the plasma membrane when the outside is acidified, it is possible that the normal penetration mechanism likewise involves a gradient of pH. Diphtheria toxin bound to the cell surface is taken up by pinocytosis and appears to be delivered to lysosomes where it is broken down to amino acids (5). The ease with which it seems to penetrate the surface membrane at low pH suggests that, after acidification of the vesicle, some of the toxin may be rapidly transferred across the vesicle membrane and thus escape digestion by lysosomal enzymes. This might occur after fusion of the toxin-bearing vesicle with a lysosome. The possibility might also be considered that the pinosomes carry a proton pump in their own membrane.

The effect of the low pH could be on the receptor molecule or on other structures in the membrane to open up some kind of "channels." Another possibility is that the low pH induces a conformational change in the toxin itself. This could expose the hydrophobic region known to be present in the B fragment (4) and facilitate its insertion into the membrane. In this connection it may be relevant that diphtheria toxin becomes highly unstable when the pH approaches the pI of the toxin (6.0) and that at high toxin concentrations it precipitates at pH 5.5 and lower (16). Interestingly, Kagan and Finkelstein showed that the incomplete diphtheria toxin crm 45, which consists of the whole A fragment and part of the B fragment, inserts itself into planar lipid bilayers. The part derived from the B fragment forms ion-permeable channels of 14-Å diameter, provided the pH is 6.0 or lower (reference 8 and personal communication from B. Kagan). Such channels would be

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sufficiently wide to allow penetration of the A fragment in its extended form. Recently Donovan et al.[1] made similar observations with whole diphtheria toxin.

The possibility has recently been considered by several authors (1, 11) that not only toxins but also various physiological molecules such as certain hormones and growth factors may enter the cytoplasm. If this occurs through "channels" in the membrane, it may be damaging to the cell to allow the channels to open up at the surface. This could permit considerable leakage of ions and intracellular molecules into the extracellular medium. Possibly, the pinocytotic vesicle acts as a "lock" into which proteins destined for the cytoplasm are taken. The signal to open the lock could be given as soon as the vesicle is well sealed off from the cell surface. In the case of diphtheria toxin, the signal appears to be a reduction in pH. With other toxins other signals may trigger channel opening.

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