Interaction of Diphtheria Toxin with Mammalian Cell Membranes*

PATRICE BOQUET$ AND ALWIN M. PAPPENHEIMER, JR.

From The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138

Uptake of $^{125}$I-labeled diphtheria toxin and serologically related proteins by a sensitive human HeLa cell line and by a resistant mouse L929 cell line has been studied. The evidence suggests that there is an initial rapid reaction between a recognition site present on the toxin Fragment B and specific plasma membrane receptors on the sensitive cell (there are approximately 4000/HeLa cell). This initial interaction is followed by a slow irreversible process during which there is a major conformational alteration of the toxin molecule causing the enzymically active 22,000-dalton Fragment A to become exposed to the cytosol. We suggest that it is at this point that cleavage of the NH$_2$ terminal disulfide bond occurs leading to release of Fragment A into the cytoplasm. The toxin Fragment B remains attached to the membrane, probably formed in a complex with receptor, and blocks entry of additional toxin molecules through the same site. Specific membrane receptors are lacking from mouse cells.

Both HeLa cells and L929 cells internalize toxin, related nontoxic proteins, and inert molecules such as inulin nonspecifically into endocytotic vesicles. At 30°C the bulk internalization of extracellular fluid is about 1.2% of their cell volume per h for both cell lines. Fragment A does not traverse the plasma membrane by a mechanism that depends on endocytosis.

The interaction of diphtheria toxin with the sensitive cell membrane is discussed in relation to other protein toxins and certain glycopeptide tropic hormones in which relatively large, hydrophilic polypeptide fragments or subunits are presumed to traverse the target cell plasma membrane and reach the cytoplasm in biologically active form.

One of the many functions of the eukaryotic cell membrane is to serve as a barrier to prevent macromolecules from reaching the cytoplasm in an intact biologically active form. Nevertheless, there are certain types of large hydrophilic molecules that possess special properties enabling them to penetrate the lipid bilayer and reach the cytosol. In viral infections, for example, it is obvious that mechanisms must exist to enable viral nucleic acid to traverse the plasma membrane. Several protein toxins, or at least large polypeptides derived from them, have been shown to reach the cytoplasm without loss of their lethal enzymic activity. Thus diphtheria toxin (1, 2), cholera toxin (3), and the toxic seed proteins, abrin and ricin (4), have each been shown to consist of an enzymically active polypeptide A, which must reach the cytoplasm to exert its lethal effect, and polypeptide B, joined to A through a disulfide bridge. In each case polypeptide B recognizes receptors on the sensitive cell, and by an as yet undetermined mechanism, allows polypeptide A to get across the plasma membrane. It seems likely that the passage of the α subunits of certain peptide hormones across the plasma membrane of their target cell may be facilitated by their β subunits by a similar mechanism (5, 6).

The diphtheria toxin molecule (62,000 daltons) is well suited for a study of the mechanism by which an enzymically active polypeptide is able to cross the plasma membrane to reach the cytoplasm. Its 22,000-dalton Fragment A is a potent enzyme (7) which, in vivo as well as in vitro, catalyzes cleavage of NAD$^+$ with the transfer of its ADP-ribose moiety to inactivate the eukaryotic polypeptide chain elongation factor, EF-2. Only a few molecules of Fragment A (perhaps only a single molecule) (8) need reach the cytoplasm in order to block protein synthesis and kill a eukaryotic cell. Nevertheless, many animal species such as rats and mice and cell lines cultured from them, are relatively resistant to the action of diphtheria toxin because they appear to lack specific membrane receptors recognized by the 38,000-dalton Fragment B. Several serologically related nontoxic proteins (CRMs) are available (9). Thus CRM197, which cannot be distinguished immunologically from toxin itself, is the product of a missense mutation

* This work was supported by Grant GB35579X from the National Science Foundation.

† Postdoctoral Fellow supported in part by French government and in part by the Institut National de la Santé et de la Recherche Médicale (INSERM).

‡ The abbreviations used are: EF-2, polypeptide chain elongation factor 2; CRM, cross-reacting material; ADP-Rib, adenine diphosphate ribose; MEM, minimal essential medium; PBS, phosphate-buffered saline; LF, flocculation unit.
that renders its Fragment A inactive, but it contains a normal Fragment B which is capable of competing with toxin for receptor sites on sensitive cell membranes (10). CRM45, on the other hand, contains a fully active Fragment A but lacks the COOH-terminal 17,000 amino acid sequence of B that is necessary for interaction with membrane receptors (11).

In the present communication, we are presenting studies on the uptake of \( ^{125}\text{I} \)-labeled diphtheria toxin, CRM197 and CRM45 by sensitive HeLa cells and by resistant mouse L-cells. The results suggest that each HeLa cell contains a limited number of specific surface receptors, lacking on mouse cells, that first react reversibly with a recognition site located, at least in part, on the 17,000-dalton COOH-terminal amino acid sequence of the toxin and CRM197 molecules. This relatively rapid, reversible reaction is followed by a slow irreversible step in which the toxin and its Fragment B become associated with receptor in the plasma membrane to block entry of additional toxin molecules through the same site. Presumably, as a result of the conformational changes involved, Fragment A is forced through the membrane. Cleavage of the disulfide bond and release of Fragment A into the cytosol is then presumed to occur at the inner surface of the membrane. It will be shown that both HeLa cells and mouse cells (although the latter are several times more resistant to the action of diphtheria toxin than HeLa cells) take up and internalize nonspecifically labeled toxin, CRM197, CRM45, and inulin within endocytotic vesicles. However, from toxin internalized in this way, little if any Fragment A actually traverses the plasma membrane to reach the cytoplasm except at exceptionally high protein concentrations.

### Materials and Methods

#### Cell Culture

- **HeLa cells**, strain S38, and mouse L929 cells were maintained in spinner cultures on MEM supplemented with 8% calf serum.

**Diphtheria Toxin and Related Proteins**—Partially purified diphtheria toxin (30 to 35% “nicked”) was from Commonwealth Laboratories, Toronto, and was further purified by DE52 chromatography (12). The final preparation had a toxicity of 25 to 30 minimum lethal dose/µg of protein and ran as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Toxin concentration was determined by flocculation against a standard antitoxin (Antitoxin and Vaccine Laboratories, Massachusetts Department of Public Health) assuming 1 Lf (floculating unit) equivalent to 2.5 µg of toxin protein. CRM45 and CRM197 were isolated and purified from culture filtrates of C7 (345) and C7/179T7, respectively, as previously described (12). All purified preparations were stored either as small aliquots in the frozen state, or as a precipitate under 0.7 saturated ammonium sulfate. In the latter case, the ammonium sulfate was removed by filtration through Sephadex G-25 just prior to use. Fragment A was prepared from diphtheria toxin by a slight modification of the method described by Michel et al. (13). Trace of Fragment B remained in the preparation as revealed by sodium dodecyl sulfate gel electrophoresis.

**Antitoxins**—For washing cells treated with \( ^{125}\text{I} \)-labeled toxin, a pepsin-treated horse γ-globulin fraction containing 2000 units/ml was used (Massachusetts Department of Health, Lot 3750). The cells were transferred to glass tubes, stirred for 1 h in the cold, and then given three more 1 ml washings with cold PBS without antitoxin. Finally, the cells were collected on glass fiber filters (Whatman GF/C) previously dipped in fetal calf serum. Cells were washed three times with 1 ml portions of cold PBS. The washed cells were then resuspended in cold PBS containing 20 units of horse diphtheria antitoxin globulin previously passed through a 0.45 µm Millipore filter. The cells were washed with cold PBS, stirred for 1 h in the cold, and then given three more 1 ml washings with cold PBS without antitoxin. Cells were then collected on glass fiber filters (Whatman GF/C) previously dipped in fetal calf serum. Cells were washed three times on the filter with cold PBS and then with 5% trichloroacetic acid and counted in a Picker γ counter.

In order to distinguish entry by specific binding from nonspecific internalization of molecules in solution through vesicle formation, suitable controls are essential. We have found that a 15- to 20-fold excess of unlabeled toxin or unlabeled CRM197 added 30 min before labeled toxin provided the most suitable control. Since endocytotic vesicle formation is negligible in the cold, uptake of label at low temperature could not be used as a control. Control cells treated with excess unlabeled toxin before addition of labeled toxin were put through the same washing procedure as described above. For estimation of the number of toxin molecules specifically bound per cell, counts were subtracted from counts of cells treated with labeled toxin only.

**Uptake of Inulin by Cell Suspensions**—The same procedure was used to measure \( ^{3}\text{H} \)-inulin uptake as described for labeled toxin except that the step in which cells were washed with antitoxin was unnecessary and ice-cold Inulin was used instead. [carboxy-\( ^{3}\text{H} \)]Inulin (New England Nuclear, 1.9 µCi/µg) was dissolved by heating in distilled water. Twenty microfilters of 0.1% solution was added to each milliliter of cell suspension. Cells were harvested, washed as described for labeled toxin uptake, and counted at hourly intervals.

**Protein Synthesis**—The rate of protein synthesis was estimated by measuring \( ^{14}\text{C} \)-lucine incorporation into trichloroacetic acid-insoluble HeLa cell protein during a 1-h pulse at 35°C (10). To each milliliter of culture, 0.2 µCi of \( ^{14}\text{C} \)-lucine (New England Nuclear) was added.

**L-[\( ^{14}\text{C} \)]Fucose-labeled Plasma Membrane Preparation from HeLa Cells**—The method of Atkinson and Summers (17), whose paper must be consulted for details, was followed. Briefly, HeLa cells were collected by centrifugation, washed once with MEM and resuspended in small spinner flasks to a cell density of 5 x 10⁶ cells/ml in 10 ml of serum-free MEM. Four microliters per ml of L-[\( ^{14}\text{C} \)]fucose (New England Nuclear, 12 Ci/mole) was then added and the suspension was stirred at 35°C for 3 h at which time toxin was added to 10⁻⁷ M. After a further hour of stirring, the intoxicated cells were collected and

---

*1 As magnetic stirring bars we use 3-mm lengths of wire cut from ordinary paper clips and sterilized by flaming.

*2 We have found horse antitoxic globulin preferable to rabbit for washing cells; perhaps because it does not form large aggregates with toxin in antitoxic excess.
Diphtheria Toxin and Membranes

Fig. 1. Distribution of iodine-125 in labeled toxin. A, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 125I-labeled toxin; 2-mm slices were counted. B, the same amount of labeled toxin was electrophoresed after treatment with 0.6% trypsin at 37°C in the presence of 1% β-mercaptoethanol. After 10 min the reaction was stopped by addition of 0.25% soy bean trypsin inhibitor.

Fig. 2. Endocytosis by HeLa cells. Washed HeLa cells were resuspended to 5 x 10^6 cells/ml (α 30 μl of packed cells) in MEM containing 2% fetal calf serum and 0.2 mg of [14C]inulin (1.9 μCi/ml), or 125I-labeled toxin as indicated below. Duplicate 1-ml samples were taken at hourly intervals and processed for counting as described under "Materials and Methods." ○, [carboxyl-14C]inulin at 30°C; x, [carboxyl-14C]inulin at 2°C; O, 125I-toxin (1.3 x 10^-7 M) + unlabeled toxin (1.6 x 10^-6 M) at 30°C; △, 125I-toxin (4 x 10^-7 M) + unlabeled toxin (8 x 10^-1 M) at 30°C.

RESULTS

Nonspecific Internalization of Extracellular Fluid—To estimate cellular uptake of molecules in solution, [carboxyl-14C]inulin uptake was measured as a function of time. As pointed out by Berger and Karnofsky (18), uptake of this inert molecule serves as a reliable measure of bulk movement of extracellular fluid into mammalian cells in culture. Fig. 2 shows that at 30°C, labeled inulin uptake by HeLa cells was linear over a 5-h period. The rate of fluid taken in by endocytosis into vesicles amounted to about 1.2% of the cell volume per h. The figure shows that the same rate of extracellular fluid internalization could be calculated from uptake of 125I-labeled toxin at two different concentrations by cells pretreated for 30 min with a large excess of unlabeled toxin. In other experiments, it was found that the nontoxic related protein, CRM45, was internalized at the same rate. Fig. 2 shows that there was relatively little uptake of extracellular fluid by cells maintained at low temperature.

The rate of inulin uptake by mouse L229 cells was also linear when followed over a 5-h period at 30°C. Within experimental error, the volume of extracellular fluid taken up per hour by mouse cells was the same as by HeLa cells whether measured by inulin uptake or by labeled toxin.

Specific Binding of Toxin by HeLa Cells—It is clear that one cannot use total uptake of label to measure specific binding of toxin to membrane receptors without correcting for nonspecific internalization by endocytosis. If the number of toxin-specific entry sites per cell is small, this correction could amount to an important fraction of the total uptake of radioactive label. Moreover, if specific membrane sites become saturated relatively quickly, as appears to be the case, we would expect that the magnitude of the correction for nonspecific uptake would continue to increase even when specific entry sites were no longer available.

Curve A of Fig. 3 shows the uptake of 125I-labeled toxin by sensitive HeLa cells following 1-h exposure at 30°C to increasing concentrations of labeled toxin. Curve B of Fig. 2 plots the number of labeled toxin molecules internalized nonspecifically into vesicles by cells pretreated for 30 min with a 20-fold excess of unlabeled toxin. By subtracting B from A we obtain the corrected Curve C for specific binding. The curve for specific binding as a function of toxin concentration is replotted in Fig. 4 which summarizes data from several experiments. The curve is hyperbolic and approaches a limiting value of close to 4200 molecules bound/cell. Approximately the same limiting value for number of toxin molecules bound per cell is approached, even at low concentrations, if binding is followed as a function of time. However, after a few hours exposure to labeled toxin, the calculation of specific binding becomes imprecise. Thus following 5-h treatment of HeLa cells with 10^-7 M toxin, the...
correction for nonspecific endocytosis approaches 90% of the total uptake of label. Even at relatively high toxin concentration, therefore, toxin does not interfere with endocytosis. A similar conclusion was drawn by Bonventre et al. (19).

The specificity of toxin binding (Figs. 3 and 4) is further supported by experiments with CRM45. As shown in Fig. 5, uptake of label by HeLa cells over a 1-h period is directly proportional to the added concentration of 125I-labeled CRM45, irrespective of whether or not a large excess of unlabeled CRM45 is present. The same is true for L-cells (Fig. 6) which show no specific binding of toxin at all. This confirms earlier studies suggesting that mouse cells are resistant to toxin because they lack specific receptors (16, 20, 21).

**Fig. 3.** Uptake of 125I-toxin by HeLa cells at 30°. HeLa cells (5 x 10⁶/ml) were treated for 1 h with increasing concentrations of labeled toxin. They were then collected and processed as described under "Materials and Methods." A, 125I-toxin; B, 125I-toxin + 20-fold excess unlabeled toxin; C, A - B = specific binding.

**Fig. 4.** Specific binding of 125I-toxin by HeLa cells at 30°. This figure summarizes the results from several experiments. The curve is drawn to fit the hyperbolic function y/(4200 - y) = [toxin] x 4 x 10⁴.

**Fig. 5.** Uptake of 125I-CRM45 by HeLa cells. HeLa cells (3 x 10⁶/ml in MEM containing 2% fetal calf serum) were treated with increasing concentrations of CRM45 for 1 h and then processed for counting in the usual manner. ○, 125I-CRM45 at 30°; ○, 125I-CRM45 + 12-fold excess of unlabeled CRM45 at 30°; x, 125I-CRM45 at 4°.

**Fig. 6.** Uptake of 131I-toxin by L-cells. L-cells were resuspended to 4 x 10⁶/ml (≈ 16 µl of packed cells) in MEM containing 2% fetal calf serum. They were treated for 1 h with toxin and then processed for counting in the usual manner. ○, 131I-toxin at 30°; ○, 131I-toxin + 12-fold excess of unlabeled toxin at 30°; x, 131I-toxin at 4°.
Previous studies (10) have shown that the nontoxic protein, CRM197, can compete with toxin for entry into HeLa cells. Fig. 7 shows that unlabeled toxin competes similarly with 125I-labeled toxin for specific binding to HeLa cell membranes. From competition experiments such as these, it was concluded that there must exist specific receptors on the sensitive cell membrane. It was suggested that these receptors interact reversibly with intact Fragment B (10). However, we have thus far been unable to obtain unequivocal evidence for reversibility, either by direct measurements at low temperature or by chase experiments using excess unlabeled toxin added 2 to 20 min after addition of labeled toxin. Although other explanations are possible, it seems reasonable to suppose that there is an initial, rapid, reversible interaction between toxin and receptor followed by a slow irreversible process. In any case it seemed worthwhile to reinvestigate the relationships between CRM197, toxin, and the cell.

Blocking of Toxin Entry Sites by CRM197—If the interaction of the toxin Fragment B with membrane receptor sites is in fact irreversible, or nearly so, then if CRM197 is allowed to interact with cells before addition of toxin, entry of toxin molecules through these sites should be blocked. Under these conditions there should no longer be any competition between CRM197 and toxin. Several lines of evidence suggest that this is indeed the case.

1. Fig. 8 plots the results of an experiment in which HeLa cells, in a series of spinner flasks, were treated with a relatively low concentration of CRM197 for 3 h in the presence of cycloheximide in order to inhibit synthesis of protein and of possible new receptor sites. They were then exposed, in the experimental Flask A, to a high saturating dose of toxin for 2 h. After washing free of excess cycloheximide, CRM197, and toxin, the cells were resuspended in fresh medium and their rate of protein synthesis was followed. The figure shows that when the cycloheximide was removed, [14C]leucine incorporation into protein resumed in all flasks, and after a brief lag, continued at its initial rate in the control Flask D to which no toxin had been added. Cells treated for 2 h with toxin alone (Flask C) or treated simultaneously for 2 h with toxin and CRM197 in a ratio of 8:1 (Flask B), incorporated [14C]leucine at about 80% the initial rate during the 1st h, but the rate fell rapidly thereafter and was only 40% of the initial rate 3 h after removing cycloheximide. On the other hand, the rate of protein synthesis in Flask A, in which cells were exposed to 5 × 10^{-7} M CRM197 for 3 h before adding 4 × 10^{-7} M toxin, declined at a significantly slower rate. As would have been expected from Figs. 2 and 3 and from our previously published studies on the kinetics of intoxication (10), the cells in Flask B behaved as if fewer than 20% of the entry sites were functional at the time toxin was added.

2. In order to obtain a rough estimate of the time required for "irreversible" blocking of entry sites by CRM197, we have carried out several experiments in which HeLa cells were first exposed at 35°C to a relatively low CRM197 concentration (4 × 10^{-4} M) and then, after increasing intervals of time, toxin was added to give a concentration of 10^{-6} M or greater. After 2 h treatment with toxin, [14C]leucine was added and its incorporation into protein measured during a 1-h pulse. The results of a typical experiment are shown in Table I. Approximately 1 h exposure to CRM197 at 35°C was required to obtain maximal blocking of toxin entry. At this time the rate of leucine incorporation was almost as great as the control without toxin and was 35 to 40% faster than when both CRM197 and toxin were added.

Fig. 8. Blockage of toxin entry into HeLa cells by CRM197. Twenty-five-milliliter HeLa cell suspensions (2.5 × 10^6 cells/ml in MEM containing 2% fetal calf serum) were placed in each of four small spinner flasks and 5 × 10^{-4} M CRM197 was added to Flasks A and D. Fifteen minutes later, 2.4 × 10^{-4} M cycloheximide, which is known not to interfere with toxin binding (22), was added to each flask. After 3 h at 35°C, 4 × 10^{-4} M diphtheria toxin was added to Flasks A and C. To Flask D was added both CRM197 and toxin to concentrations of 5 × 10^{-4} M and 4 × 10^{-4} M, respectively. Flask D served as a control. After two more hours at 35°C, cells from each flask were collected, washed once with medium in the centrifuge to remove cycloheximide, excess CRM197, and toxin, and were finally resuspended in medium containing 1 unit/ml of diptheria antitoxin. At intervals, samples were withdrawn from each flask and pulse-labeled with [U-14C]leucine for 1 h. 1. CRM197 for 3 h, then toxin for 2 h (Flask A); O, CRM197 and toxin together at 3 h (Flask B); X, toxin only at 3 h (Flask C); A, Control; no toxin (Flask D). Note: after addition of cycloheximide, protein synthesis fell to 15 to 20% or less in all flasks.
were added at the same time. Longer incubation with CRM197 failed to increase the inhibition further; indeed, there was some decrease after 2 h probably because of degradation of CRM197.

3. More direct evidence that exposure to CRM197 prevents subsequent binding of toxin is shown in Table II. HeLa cells were pretreated with 4 \times 10^{-8} \text{ M CRM197} for 2 h at 30^\circ \text{C} and washed before adding 10^{-8} \text{ M } ^{131}\text{I}-\text{labeled toxin. The table shows that under these conditions, only about 50% as much label was bound to the cells as with toxin alone. When the same concentrations of CRM197 and toxin were added simultaneously, there was only a slight decrease in the amount of toxin bound. Table II shows that under the same conditions, L-cells behaved very differently and there was no effect of CRM197 on toxin internalization into vesicles by L-cells either when added before or together with labeled toxin.

Effect of Temperature—In Fig. 9, we have plotted the effect of temperature on uptake of toxin by HeLa cells and by L-cells after 20 min treatment with 10^{-7} \text{ M } ^{131}\text{I}-\text{labeled toxin. The uptake of label below 15^\circ \text{C} is at approximately the same low level for cells of both species. The relative difference between the two species becomes most apparent between 15^\circ \text{C} and 25^\circ \text{C} due to specific binding by HeLa cells. Above 25^\circ \text{C}, uptake due to endocytosis increases very rapidly in both species so that the relative difference between them is less striking. For this reason, we have carried out all our binding experiments at the intermediate temperature of 30^\circ \text{C}.

Does Diphtheria Toxin Fragment B Reach Cytoplasm?—Intact diphtheria toxin has very little enzymic activity (23, 24). Activation requires reduction of the disulfide bridges and hydrolysis of a peptide bond in the exposed loop subtended by the NH_{2}-terminal disulfide bridge. It is only necessary for a few molecules of Fragment A of toxin to reach the cytosol in order to block polypeptide chain elongation. What is the fate of Fragment B? Previous autoradiographic studies on sections from HeLa cells treated with ^{131}\text{I}-labeled toxin suggested that most of the label remained fixed to the plasma membrane (16).

In order to identify membrane-associated toxin-derived material, we first attempted to react toxin-treated HeLa cells with an avid rabbit antitoxin covalently bound to Sepharose 4B. Despite the fact that almost all of the antibodies in this serum were directed against determinants on Fragment B (12), we were unable to demonstrate binding of toxin-treated cells to Sepharose-bound antitoxin, nor could we detect agglutination of toxin-treated cells by soluble antitoxin. This suggested that the toxin or its Fragment B had changed in conformation or position on the membrane so that their antigenic determinants were no longer available for interaction with antitoxin.

Although we could obtain no evidence that antitoxin can react with toxin that has already become bound to the living cell membrane, plasma membrane preparations from toxin-treated cells do react with antitoxin as shown by the following experiment. HeLa cells were first labeled with L-[\text{35S}]fucose, which is known to be incorporated almost exclusively into plasma membrane glycoproteins (17), and then the labeled cells were treated with 10^{-7} \text{ M} toxin for 1 h at 35^\circ \text{C}. The cells were then thoroughly washed in the usual manner except that the washing with antitoxin was omitted. Instead, the cells were finally stirred for 2 h at 10^\circ \text{C} with phosphate-buffered saline. Purified membranes prepared from these washed, intoxicated cells containing the fucose marker were incubated with an antitoxin specific for Fragment B that had been covalently bound to Sepharose 4B beads. As seen from Table III, the labeled membranes became fixed to the immunosorbent. In the presence of an excess of soluble toxin, however, the binding was markedly decreased. That the interaction involved antibodies specific for determinants on Fragment B is indicated by the fact that even a large excess of Fragment A had only a slight inhibitory effect. This slight inhibition could be accounted for by traces of Fragment B still remaining in the preparation used.

**DISCUSSION**

Our studies suggest that the process whereby the 22,000-dalton, enzymically active Fragment A of diphtheria toxin traverses the plasma membrane of sensitive cells to reach the cytoplasm, is a highly specific one. We propose that the entry process involves successive steps as follows.

1. In the case of HeLa cells, each cell contains on its surface,
About 4,000 specific membrane receptors that react reversibly with sites located on Fragments B of toxin or CRM197. Evidence suggesting that this initial reaction may be reversible comes from the fact that CRM197, which contains normal Fragment B, will compete freely with toxin for receptor sites (10). We have now shown similar competition between labeled and unlabeled toxin. Quantitative studies of the competition between toxin and CRM197 enabled Ittelson and Gill (25) to calculate an apparent binding constant of 10^8 liters/mol for CRM197. Since CRM45 fails to bind to HeLa cells, any specific determinants involved are presumably located, at least in part, on the 17,000-dalton COOH-terminal amino acid sequence of Fragment B. This reversible reaction is probably relatively insensitive to temperature changes, but specific evidence bearing on this point is lacking.

2. The initial reaction is followed by a slow irreversible process during which receptors become inactivated or at least unavailable for reaction with additional toxin molecules. The entry sites are therefore blocked. We suggest that during this process, toxin molecules or their Fragments B may become imbedded in the plasma membrane, perhaps in association with a membrane receptor protein. The process does not take place below 15-20°C. In any event, the interaction must certainly involve major conformational changes in the toxin molecule so that hydrophobic groups of the normally hydrophilic protein can interact and so that the disulfide bridge joining Fragment A to B can reach the inner membrane surface.

3. Finally, cleavage of the disulfide bridge takes place and in the case of "nicked" toxin, Fragment A is released into the cytoplasm leaving B behind as a membrane protein. Whether hydrolysis of the peptide linkage joining the two fragments in intact toxin also takes place at this time, or whether the Fragment A which reaches the cytosol is derived only from toxin nicked before interaction with the cell, is not clear.

It has generally been supposed that protein molecules which succeed in reaching the cytoplasm in a biologically active form, do so by an endocytotic process (26). This does not seem to be the case with diphtheria toxin. Nevertheless, when considering uptake of labeled hydrophilic proteins by cells, it is important to distinguish between mere internalization by an endocytotic process and actual traversal of the lipid bilayer into the cytosol. In the former case, endocytotic vesicles may fuse with lysosomal granules where entrapped proteins may become degraded but do not ordinarily cross the plasma membrane in an intact form. If the concentration of entrapped molecules is sufficiently high, however, and if some fused vesicles rupture and release their contents before degradation is complete, occasional relatively resistant molecules (such as the Fragment A of diphtheria toxin) may reach the cytoplasm while still in an enzymatically active form.

There is no doubt that diphtheria toxin can become internalized nonspecifically by cultured cells, even cells from resistant species that lack specific membrane receptors. This was suggested by Moehring and Moehring (20) from their studies on the action of toxin on mouse cells. They showed that polyornithine, which is known to enhance pinocytotic activity, would increase the sensitivity of L-cells severalfold. Bonventre et al. (19), from their experiments with 125I-labeled toxin and mouse L-cells, concluded that cellular uptake of toxin by endocytosis does not necessarily mean cell destruction.

In the present experiments, we have used labeled inulin to measure the bulk uptake of extracellular fluid into endocytotic vesicles. We have found that mammalian cell suspensions in spinner flasks take up 1.2% or more of their cell volume per hour at 30°C. The same rate of fluid uptake could be calculated...
Diphtheria Toxin and Membranes

Refines et al. (28) have proposed that endocytosis is involved in the mechanism whereby the toxic seed proteins abrin and ricin enter mammalian cells to block protein synthesis. These proteins first react, via their B chains, with glycoprotein surface receptors containing terminal nonreducing galactose units (29). Refines et al. have suggested that before their A chains can cross the membrane, the bound toxins must first be internalized by endocytosis. Nicolson et al. (30) used actively endocytotic lymphoma cells treated with a ferritin-ricin, (RCA) conjugate to visualize directly in the electron microscope the toxic protein conjugate bound to the plasma membrane within vesicles. Using high concentrations of the conjugate, they were able to detect some ferritin molecules within the cytoplasm that appeared to have escaped from ruptured vesicles. However, a few simple calculations will show that such a mechanism cannot explain the passage of diphtheria toxin Fragment A across the plasma membrane. It is known that extracts of mouse L-cells are just as sensitive as HeLa cell extracts to the enzymic activity of Fragment A (20, 31). Yet at least 5 h exposure at 37°C to a 10^{-5} M concentration of diphtheria toxin is required to reduce protein synthesis in growing L-cells by 50%. From their extracellular fluid uptake, it can be calculated that at least 2 x 10^6 toxin molecules would be internalized per cell after 5 h at this concentration. On the other hand, only 10^{-6} M or less toxin is required to produce the same effect on HeLa cells and this would correspond to an endocytotic uptake of only about 200 molecules/cell. If 2 x 10^4 molecules were really required to kill a HeLa cell, we would have to assume that each cell had at least 2 x 10^4 receptors with an affinity for toxin higher than 10^9 liters/mol. This clearly cannot be the case. From the equation describing specific binding (see Fig. 3) it may be calculated that after 1 h treatment with 10^{-8} M toxin at 30°C, about 160 molecules are specifically bound per HeLa cell or 800 in 5 h. If this were the maximum number of molecules that must reach the cytoplasm in the case of L-cells to reduce protein synthesis by 50%, then the chance of a given molecule escaping degradation to reach the cytoplasm would be only 1 in 2000 or less. Such a mechanism becomes even more improbable in the case of abrin or ricin where a concentration of less than 10^{-11} M suffices to reduce protein synthesis in either mouse or human cells by 50% within 3 h (32).

Do other biologically active peptides reach the eukaryotic cell interior by mechanisms similar to that involved in traversal of Fragment A of diphtheria toxin across the cell membrane? Recent studies on the structure and mode of action of cholera toxin have shown that, like diphtheria toxin, its molecule consists of an enzymically active fragment A, linked to a recognition B unit via a short polypeptide chain, A, which is joined to A by a disulfide bridge (3, 33). Polypeptide A brings about the enzymatic activation of adenylyl cyclase (34, 35). Gill (36-38) has shown that activation of the cyclase requires several cytoplasmic factors and A must therefore reach the cytosol itself, in order to exert its action. As with diphtheria toxin, the cholera B moiety (choleragenoid) is needed both for recognition of surface receptors and probably to facilitate traversal of A across the membrane. Isolated cholera toxin A and choleragenoid are both nontoxic. As is the case with diphtheria toxin and CRM197, interaction of a limited number of receptor sites with choleragenoid (or with toxin itself) prevents further interaction with intact toxin (39, 40).

The interaction of the toxic seed proteins, abrin and ricin, with cell membranes would also appear to follow the diphtheria toxin model. The A chains of these toxins must reach the cytoplasm in order to arrest protein synthesis by inactivation of the 60 S eukaryotic ribosomal subunit (4). Again, a B chain is linked to A through a disulfide and is required for interaction with receptors and passage of the A chain across the cell membrane. Several other toxic proteins have been reported to contain polypeptide A chains joined to B chains by disulfide bridges; tetanus toxin (41, 42), botulinus toxin (43), staphylococcus “B” toxin (44), but as yet little is known of their mode of action. As final examples, we may cite recent work on interaction of low density lipoproteins and of certain glycoprotein tropic hormones with membrane receptors. The specific and nonspecific uptake of low density lipoproteins by cultured fibroblasts has been shown by Goldstein and Brown (45) to follow kinetic data very similar to those we are now reporting for diphtheria toxin. Thyroid-stimulating hormone, luteinizing hormone, and chorionic gonadotropin each consist of α and β subunits (5) which, as pointed out elsewhere (6), appear to be analogous in function to toxin polypeptides A and B. Thus the isolated hormone α and β subunits are biologically inactive. Complete amino acid sequence analysis has shown that human thyroid-stimulating hormone, luteinizing hormone, and chorionic gonadotropin α subunits contain nearly identical primary structures, whereas the corresponding β subunits which confer target cell specificity appear to be far more variable in primary sequence (5). As with abrin and ricin (6) and the proteins related to diphtheria toxin, CRM45 and CRM197, isolated α and β subunits from the three hormones can recombine to yield fully active hybrid molecules, the specificity of which is determined by their β subunit (5). It is tempting to conclude that the α subunits of these hormones must cross the membrane in order to activate their target cell. In order to reach the cell interior, specific receptors of the target cell must first interact with the β subunit which then promotes passage of α across the plasma membrane by a process analogous to that by which diphtheria toxin Fragment A reaches the cytoplasm. Very recently, Ketelsleegers et al. (46), have studied the kinetics of 125I-labeled chorionic gonadotropin binding by rat testis cells. From their studies, there would appear to be an initial reversible interaction between hormone and receptors on the target cells, followed by a slow temperature-sensitive irreversible step during which the receptors become inactive and no longer bind labeled hormone. Once again, the similarity to the kinetics of diphtheria toxin interaction is striking.

Acknowledgments—We are grateful to Dr. Guido Guidotti and to D. M. Gill for their critical reading of the manuscript and to Ms. Vickery Randall for valuable technical assistance.

REFERENCES
1. Gill, D. M., and Dinius, L. L. (1971) J. Biol. Chem. 246, 1485-1491
2. Collier, R. J., and Kandel, J. (1971) J. Biol. Chem. 246, 1496-1503
3. Finkelstein, R. A. (1973) Crit. Rev. Microbiol. 3, 553-623
4. Olness, S., Refnes, K., and Pihl, A. (1974) J. Immunol. 113, 842-847
5. Tager, H. S., and Steiner, D. F. (1974) Annu. Rev. Biochem. 43, 551
6. Olness, S., Pappenheimer, A. M., Jr., and Meren, R. (1974) J. Immunol. 113, 842-847
Diphtheria Toxin and Membranes

7. Pappenheimer, A. M., Jr., and Gill, D. M. (1973) Science 182, 353-358
8. Gill, D. M., Pappenheimer, A. M., Jr., and Uchida, T. (1973) Fed. Proc. 32, 1508-1515
9. Uchida, T., Pappenheimer, A. M., Jr., and Greany, R. (1973) J. Biol. Chem. 248, 3838-3844
10. Uchida, T., Pappenheimer, A. M., Jr., and Harper, A. A. (1973) J. Biol. Chem. 248, 3845-3850
11. Uchida, T., Gill, D. M., and Pappenheimer, A. M., Jr. (1971) Nature New Biol. 233, 8-11
12. Pappenheimer, A. M., Jr., Uchida, T., and Harper, A. A. (1972) Biochemistry 9, 891-906
13. Michel, A., Zanen, J., Monier, C., Crispell, C., and Dirkx, J. (1972) Biochim. Biophys. Acta 257, 249-256
14. Axén, R., Forath, J., and Ernback, S. (1967) Nature 214, 1302-1304
15. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) Biochem. J. 89, 114-123
16. Pappenheimer, A. M., Jr., and Brown, R. (1968) J. Exp. Med. 127, 1073-1086
17. Atkinson, P. H., and Summers, D. F. (1961) J. Biol. Chem. 246, 5162-5175
18. Berger, R. R., and Karnovsky, M. L. (1966) Fed. Proc. 25, 840-845
19. Bonventre, P. F., Saelinger, C. B., Ivins, B., Woschinski, C., and Amorini, H. (1975) Infect. Immun. 11, 675-684
20. Moehring, J. M., and Moehring, T. J. (1968) J. Exp. Med. 127, 533-541
21. Creagan, R. P., Chen, S., and Ruddle, F. H. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2237-2241
22. Gill, D. M., Pappenheimer, A. M., Jr., and Baseman, J. B. (1969) Cold Spring Harbor Symp. Quant. Biol. 34, 595-602
23. Gill, D. M., and Pappenheimer, A. M., Jr. (1971) J. Biol. Chem. 246, 1492-1495
24. Drazin, R., Kandel, J., and Collier, R. J. (1971) J. Biol. Chem. 246, 1504-1510
25. Ittelson, T. R., and Gill, D. M. (1973) Nature 242, 330-332
26. Ryser, H. J. P. (1968) Science 160, 390-396
27. Steinman, R. M., Silver, J. M., and Cohn, Z. A. (1974) J. Cell. Biol. 63, 949-969
28. Refanes, K., Olsnes, S., and Pihl, A. (1974) J. Biol. Chem. 249, 3557-3562
29. Adair, W. L., and Kornfeld, S. (1974) J. Biol. Chem. 249, 4696-4704
30. Nicolson, G. L., LaCorbiere, M., and Hunter, T. R. (1975) Cancer Res. 35, 144-155
31. Goor, S., Pappenheimer, A. M., Jr., and Ames, E. (1967) J. Exp. Med. 126, 925-930
32. Pappenheimer, A. M., Jr., Olsnes, S., and Harper, A. A. (1974) J. Immunol. 113, 835-841
33. Van Heyningen, S. (1974) Science 183, 666-667
34. Field, M. (1971) N. Engl. J. Med. 284, 1137-1144
35. Sharp, G. W. G., and Hynie, S. (1971) Nature 229, 266-269
36. Gill, D. M., and Klug, C. (1975) J. Biol. Chem. 250, 4924-4932
37. Gill, D. M. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 2064-2068
38. Gill, D. M. (1976) J. Infect. Dis. 133, Suppl. S56-63
39. Cuatrecasas, P. (1973) Biochemistry 12, 3556-3566
40. Pierce, N. F. (1973) J. Exp. Med. 137, 1009-1023
41. Caven, C. J., and Dawson, D. J. (1973) Biochim. Biophys. Acta 317, 271-275
42. Matsuda, M., and Yoneda, M. (1974) Biochem. Biophys. Res. Commun. 53, 1297-1292
43. Sugiyama, H., DasGupta, B. R., and Yang, K. H. (1973) Proc. Soc. Exp. Biol. Med. 143, 589-591
44. Spero, L., Metzger, J. P., Warren, J. R., and Griffin, B. Y. (1975) J. Biol. Chem. 250, 5026-5032
45. Goldstein, J. L., and Brown, M. S. (1974) J. Biol. Chem. 249, 5148-5159
46. Ketelslegers, J.-M., Knot, G. D., and Catt, K. S. (1975) Biochemistry 14, 3075-3082
Interaction of diphtheria toxin with mammalian cell membranes.

P Boquet and A M Pappenheimer, Jr

J. Biol. Chem. 1976, 251:5770-5778.

Access the most updated version of this article at http://www.jbc.org/content/251/18/5770

Alerts:
  - When this article is cited
  - When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/251/18/5770.full.html#ref-list-1