The receptor-mediated internalization and degradation of radiolabeled diphtheria toxin by cultured monkey kidney cells was studied. The ability of a number of enzymes and chemicals to remove cell surface-bound toxin was tested; the combination of pronase and inositol hexaphosphate (PIHP) proved most effective. Using PIHP, the kinetics of toxin-cell association at 37°C was resolved into two components: surface binding and internalization. The PIHP assay also allowed estimation of the half-time of toxin internalization (about 25 min). An assay involving precipitation of culture supernatants with trichloroacetic acid was developed and used to measure the rate of degradation and excretion of cell-associated toxin. Agents which markedly inhibited toxin internalization similarly prevented degradation, implying an intracellular location for the degradative process. The primary radioactive product excreted by Vero cells was monoiodotyrosine. The extent and rate of toxin degradation indicated lysosomal involvement. Finally, agents which blocked internalization or degradation, or both, (e.g. antibody and concanavalin A), protected cells from the cytotoxic action of diphtheria toxin, suggesting that these processes are necessary for expression of biological effect.

It has been recently shown that diphtheria intoxication of highly sensitive mammalian cell lines is a receptor-mediated process (1); that is, the intracellular events culminating in cytotoxicity are preceded by the binding of toxin to specific cell-surface receptors. The intracellular mode of action of diphtheria toxin has been extensively characterized (2, 3): an enzymatically active portion of the toxin, Fragment A, catalyzes the transfer of the adenosine diphosphoribose moiety of NAD to elongation factor 2, an essential translocase involved in eukaryotic protein synthesis. Adenosine diphosphoribose elongation factor 2 is metabolically inactive and subsequent cell death is directly attributable to inhibition of intracellular protein synthesis. The means by which the toxin or toxin-receptor complex is internalized by the cell is as yet unclear. Recent studies have shown that many macromolecules, including insulin (4), human chorionic gonadotropin (5), and EGF (6), are rapidly internalized by cells. However, there is little direct evidence that the biological function of those molecules requires internalization. On the other hand, it is obvious that at least the active fragment of diphtheria toxin must enter the cell in order to effect the ADP-ribosylation of elongation factor 2. Thus, internalization in this case is a prerequisite for expression of biological activity.

In this sense, diphtheria toxin is more analogous to LDL, a cholesterol-carrying serum macromolecule whose metabolic effects are exerted through a receptor-mediated internalization and degradation process (7). Degradation of LDL occurs in secondary lysosomes and results in the release of free cholesterol into the cytoplasm. Similarly, diphtheria toxin must also undergo some form of degradation (or fragmentation) to express its biological activity. Toxin which is either intact or has a cryptic peptide bond cleavage (“nicked” toxin) is enzymatically inactive in vitro (3) but is cytotoxic to cells and animals in vivo. Enzymatically active Fragment A must, therefore, be released from whole toxin during cellular intoxication subsequent to the binding of toxin to receptor.

As shown by our previous studies, the kinetic response of sensitive cells to 125I-labeled diphtheria toxin at 37°C is biphasic, reaching a peak at 1 to 2 h and decreasing thereafter to a steady state approximately 50% of the maximum (1). It seemed probable that this pattern resulted from sequential cell-surface binding, internalization, and intracellular processing of the 125I-labeled toxin. At physiological temperature, however, it is difficult to quantitate the degree of cell-surface binding since total cell-associated radioactivity presumably represents both surface-bound and internalized material. In this investigation, we have developed a technique which permits such quantitative studies and have defined the relationship between surface binding and subsequent intracellular uptake and processing.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture—**Seed stock for the Vero and BS-C-1 cell lines was obtained from the American Type Culture Collection (ATCC). The lines were maintained in 75-cm² T-flasks (Costar No. 3075) or 700-cm² roller bottles (Costar No. 1234) with the medium and serum supplement recommended by ATCC.

**Media and Sera—**All media, vitamins, antibiotics, and amino acids were obtained from Grand Island. Fetal calf serum was purchased from Reheis and heat-inactivated for 30 min at 56°C before use in cell culture experiments.

**Materials—**“Low pH” carrier-free Na 125I was obtained from New England Nuclear. Pronase (B grade, Catalog no. 537088), heparin, and methyl-a-D-mannopyranoside were purchased from Calbiochem; trypsin (180 unit/g) and collagenase (Type IV, 150 unit/250 units/mg) were obtained from Worthington Biochemical Corp. Inositol hexaphosphate was obtained from P-L Biochemicals; dextran sulfate and concanavalin A were from Pharmacia. Adenosine 5’-triphosphate (ATP) and 3-iodo-L-tyrosine (moniodotyrosine) were purchased from Sigma. All other chemicals were reagent grade and were used without further purification.

**Toxin—**Diphtheria toxin was obtained from Connaught Labora-
Internalization and Degradation in a model automatic counting system (Searle Analytic, Inc.). The monolayers then were rinsed four times with Hanks' balanced salt solution (HBSS), solubilized in 1.0 ml of 0.1 M NaOH, and counted in a model 1185 automatic γ counting system (Searle Analytic, Inc.).

The difference between binding of labeled toxin in the presence and absence of excess unlabeled toxin represents specific binding; the data presented in this paper are shown in the form of specific counts. The level of nonspecific binding in all cases represented less than 10% of the total.

Internalization Assay—Cell monolayers were incubated with 125I-labeled diphtheria toxin with or without a 100-fold excess of unlabeled toxin as described above. The monolayers then were rinsed three times with cold HBSS and incubated 60 min at 4°C with 0.5 ml of pronase (0.25 mg/ml) plus inositol hexaphosphate (IHP) (10 mg/ml) in HBSS. At the end of the incubation period, 0.5 ml of 100% heat-inactivated FCS was added to each well, the wells were scraped, and the detached cells were transferred to 1.5-ml polypropylene centrifuge tubes and spun for 1 min in a microfuge (Eppendorf model 5412). Both pellets and supernatants were counted as above.

Release of Trichloroacetic Acid-soluble Fragment Release—Cell monolayers were incubated with 125I-labeled diphtheria toxin with or without a 100-fold excess of unlabeled toxin at 4°C for the times indicated. The monolayers then were rinsed three times with cold HBSS and incubated 60 min at 4°C with 0.5 ml of pronase (0.25 mg/ml) plus IHP (10 mg/ml) in HBSS. At the end of the incubation period, the monolayers were washed rapidly with HBSS and incubated with 1 ml/well of complete Hanks' 199 supplemented with 1% FCS, 25 mM Hepes buffer, pH 7.4. At this point, the culture supernatant was collected and centrifuged to remove any detached cells, and moniodotyrosine (100 μmol in 50% acetone) was added as carrier. Samples were lyophilized, redissolved in 1 ml of water, and precipitated by addition of trichloroacetic acid (10%). Approximately 50% of the original cell-associated radioactivity was removed by this extraction. The supernatant was diluted to 10 ml in 0.02 M HCl and adsorbed to an 0.8-ml bed of Dowex 50 X-2. The column was washed with 0.01 M acetic acid and eluted with 0.15 M NH4OH. The radioactivity fractions (13,000 cpm) were pooled and lyophilized. One-tenth of the reconstituted sample was spotted on a silica gel thin layer plate and the chromatograms developed with 1-butanol/acetic acid/H2O (10:1:1).

Release of diphtheria toxin from the cell surface by various compounds

Vero cells were bound for 18 h at 4°C with 0.03 μg/ml of 125I-labeled diphtheria toxin. The monolayers then were rinsed three times with HBSS and incubated for 1 h at 4°C in the presence of releasing agent at the concentrations listed. FCS then was added to a final concentration of 50%, the wells were scraped with a small Teflon scraper, and triplicate samples were processed as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Compound</th>
<th>Radioactivity released (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of initial cell-bound specific radioactivity</td>
</tr>
<tr>
<td>HBSS (control)</td>
<td>10</td>
</tr>
<tr>
<td>Trypsin (2.5%)</td>
<td>35</td>
</tr>
<tr>
<td>+EDTA (0.2 g/liter)</td>
<td>26</td>
</tr>
<tr>
<td>+IHP (10 mg/ml)</td>
<td>76</td>
</tr>
<tr>
<td>Collagenase (1.0%)</td>
<td>14</td>
</tr>
<tr>
<td>Alone</td>
<td>88</td>
</tr>
<tr>
<td>+EDTA (0.2 g/liter)</td>
<td>84</td>
</tr>
<tr>
<td>+IHP (10 mg/ml)</td>
<td>89</td>
</tr>
<tr>
<td>Pronase (0.25%)</td>
<td>88</td>
</tr>
<tr>
<td>Alone</td>
<td>88</td>
</tr>
<tr>
<td>+EDTA (0.2 g/liter)</td>
<td>84</td>
</tr>
<tr>
<td>+IHP (10 mg/ml)</td>
<td>89</td>
</tr>
<tr>
<td>IHP (10 mg/ml)</td>
<td>88</td>
</tr>
<tr>
<td>Alone</td>
<td>43</td>
</tr>
<tr>
<td>+EDTA (0.2 g/liter)</td>
<td>39</td>
</tr>
<tr>
<td>EDTA (0.2 g/liter)</td>
<td>11</td>
</tr>
<tr>
<td>Heparin (10 mg/ml)</td>
<td>14</td>
</tr>
<tr>
<td>Dextran sulfate (10 mg/ml)</td>
<td>19</td>
</tr>
<tr>
<td>Sodium sulfate (10 mg/ml)</td>
<td>12</td>
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<tr>
<td>Concanavalin A (100 μg/ml)</td>
<td>9</td>
</tr>
<tr>
<td>Methyl-α-D-mannopyranoside (0.2 M)</td>
<td>9</td>
</tr>
<tr>
<td>ATP (1 mM)</td>
<td>12</td>
</tr>
<tr>
<td>Diphtheria toxin (3 μg/ml)</td>
<td>19</td>
</tr>
</tbody>
</table>
in the l-h time period of the experiment. In the presence of diphtheria toxin with Vero cells. The kinet-
amic acid from cells treated with PIHP in the experiments using an established procedure (11) showed no
apparent plasma membrane damage as assessed by leakage of manner described. The results shown in Table I and Fig. 1 led us to adopt a l-h incubation in PIHP as a standard assay for
over 85% of the total was removed from the cells. Control
radioactivity were released into the medium until, at 60 min,
PIHP, however, increasing amounts of the cell-associated
radioactivity; 0, PIHP-releasable radioactivity; 0, PIHP-resistant
cell-associated radioactivity remains essentially constant over
the 1-h time period of the experiment. In the presence of PIHP, however, increasing amounts of the cell-associated radioactivity were released into the medium until, at 60 min, over 85% of the total was removed from the cells. Control
experiments using an established procedure (11) showed no apparent plasma membrane damage as assessed by leakage of [14C]aminobutyric acid from cells treated with PIHP in the manner described. The results shown in Table I and Fig. 1 led us to adopt a 1-h incubation in PIHP as a standard assay for
cell surface-bound toxin.

**Kinetcs of Toxin Internalization**—Development of the PIHP method allows detailed examinations of the nature of the association of diphtheria toxin with Vero cells. The kinetics of toxin internalization as assayed by the PIHP technique was followed at 4 and 37°C. Results are shown in Fig. 2. Cells were equilibrated to either 4 or 37°C and [125I]-toxin added at zero time. Samples were harvested either using PIHP or by solubilization in NaOH as described for the binding assay under “Experimental Procedures.” At 4°C, PIHP-releasable radioactivity continued to increase in the presence of [125I]-labeled toxin and at all time points represented approximately 85% of the total cell-associated radioactivity. This indicates that essentially all of the [125I]-labeled toxin remained on the cell surface. Very little radioactivity was associated with the cell pellet. At 37°C, however, the increase in PIHP-releasable radioactivity was accompanied by an increase in PIHP-resistant (pellet) radioactivity. Both reached a maximum level around 1 to 2 h after toxin addition and decreased thereafter; PIHP-releasable (surface-bound) counts decreased more rapidly than PIHP-resistant (internalized) counts. After 1 to 2 h, approximately 50% of the total cell-associated radioactivity was resistant to release by PIHP. This experiment demonstrates that the biphasic kinetic curve exhibited by Vero cells at 37°C can be resolved into two components using the PIHP technique: total cell-associated radioactivity is the sum of PIHP-releasable (surface-bound) and PIHP-resistant (internalized) radioactivity.

**Half-time of Toxin Internalization**—Since it is difficult to determine the half-time of toxin internalization from experiments of the type shown in Fig. 2, additional experiments were performed using cells prebound with [125I]-labeled diphtheria toxin at 4°C (Fig. 3). At zero time, following a 12-h incubation at 4°C, essentially all of the cell-associated radioactivity represents surface-bound diphtheria toxin and is fully releasable by PIHP. At this point the cells were rinsed three times to remove unbound toxin and the incubation was continued at 4 or 37°C. At 4°C, the slight decrease in PIHP-releasable radioactivity was due to dissociation of surface-bound toxin from its receptor. There was no detectable in-
crease in PIHP-resistant radioactivity. With continued incubation at 37°C, PIHP-releasable radioactivity decreased markedly over the period of the experiment with a concomi-
tant increase in PIHP-resistant activity, representing internal-
ized toxin. From these data, the half-time of internalization of diphtheria toxin by Vero cells was estimated to be 25 min.

The concentration of [125I]-labeled toxin used in these studies (0.03 μg/ml) has been shown to saturate approximately 10 to

**Fig. 1. Kinetics of diphtheria toxin removal from the cell surface by PIHP.** [125I]-toxin (0.03 μg/ml) was added to the cells for 18 h at 4°C. Cells then were rinsed three times with HBSS, PIHP (0.25 mg/ml of pronase + 10 mg/ml of IHP) was added, and incubations were carried out for the times indicated at 4°C. PBS then was added to a final concentration of 30%, the wells were scraped with a small Teflon scraper, and triplicate samples were processed as described under “Experimental Procedures.” ▲, total cell-associated radioactivity; □, PIHP-releasable radioactivity; ○, PIHP-resistant radioactivity.

**Fig. 2. Kinetics of diphtheria toxin internalization.** [125I]-toxin (0.03 μg/ml) was added to the cells at either 37°C (A) or 4°C (B). At the times indicated, triplicate samples were assayed for total cell-associated radioactivity or for internalization as described under “Experimental Procedures.” ●, total cell-associated radioactivity; □, PIHP-releasable radioactivity; △, PIHP-resistant radioactivity.

**Fig. 3. Half-time of internalization of diphtheria toxin.** [125I]-toxin (0.03 μg/ml) was added to the cells for 18 h at 4°C. Cells then were rinsed three times with HBSS, fresh complete medium was added, and incubation was continued at either 4 or 37°C. At the times indicated, triplicate samples were processed as described under “Experimental Procedures.” At 4°C: ●, PIHP-releasable radioactivity; □, PIHP-resistant radioactivity. At 37°C: ●, PIHP-releasable radioactivity; ■, PIHP-resistant radioactivity.
Experimental Procedures. At 4°C: total cell-associated radioactivity; at 37°C: total cell-associated radioactivity; trichloroacetic acid-soluble radioactivity. At 37°C: total cell-associated radioactivity; trichloroacetic acid-soluble radioactivity. At 4°C: total cell-associated radioactivity; trichloroacetic acid-soluble radioactivity. 

FIG. 4. Kinetics of diphtheria toxin degradation. ¹²⁵I-toxin (0.03 μg/ml) was added to the cells for 18 h at 4°C. Cells then were rinsed three times with HBSS, fresh complete medium was added, and incubation was continued at either 4°C or 37°C. At the times indicated, triplicate samples were processed as described under ‘Experimental Procedures.’ At 4°C: [] total cell-associated radioactivity; ■, trichloroacetic acid-soluble radioactivity. At 37°C: ○, total cell-associated radioactivity; ●, trichloroacetic acid-soluble radioactivity.

FIG. 5. Characterization of trichloroacetic acid-soluble radioactivity. ¹²⁵I-toxin (0.1 μg/ml) was added to cell monolayers in 75-cm² T-flasks for 1 h at 37°C. The cells then were rinsed three times with HBSS, fresh medium was added, and incubation was continued for 4 h at 37°C. The culture supernatant was collected and processed as described under ‘Experimental Procedures.’ The position of the carrier 3-iodo-L-tyrosine (MIT), visualized by ninhydrin spray, is shown for comparison.

20% of the available surface receptor sites yielding 10,000 to 20,000 toxin molecules bound/cell (1). Calculations based on the data shown in Fig. 3 show that approximately 10,000 toxin molecules were internalized/cell.

Degradation of Internalized Toxin—As shown in Fig. 3, when cells were prebound with ¹²⁵I-labeled diphtheria toxin at 4°C and subsequently rinsed and warmed at 37°C, total cell-associated radioactivity decreased rapidly to about 20% of the initial level. The decrease was hypothesized to result from the internalization, intracellular degradation, and subsequent excretion of toxin fragments by the cells. This hypothesis was supported by the data shown in Fig. 4. When cells were prebound with ¹²⁵I-labeled diphtheria toxin at 4°C, rinsed, and transferred to 37°C, the ¹²⁵I-toxin was rapidly degraded into trichloroacetic acid-soluble fragments. The amount of trichloroacetic acid-soluble radioactivity in the culture supernatant increased with time of incubation at 37°C to a plateau level around 3 to 4 h, at which point approximately 60 to 70% of the original cell-associated radioactivity had been excreted in degraded form. About 10% of the original cell-associated radioactivity was detectable in the culture supernatants in trichloroacetic acid-precipitable form.

Degradation experiments of this type were performed using prebound, rinsed cells because the ¹²⁵I-labeled diphtheria toxin preparation contained a persistent contaminating amount of trichloroacetic acid-soluble radioactivity (approximately 3 to 5%) which complicated the detection of cell-dependent fragment excretion. The rinse procedure effectively eliminated this problem. Chloroform extraction of culture supernatants incubated in this manner did not demonstrate measurable free iodine (12).

Characterization of Trichloroacetic Acid-soluble Fragments—The nature of the trichloroacetic acid-soluble material released by the cells at 37°C was investigated using thin layer chromatography. The chromatogram in Fig. 5 demonstrates that the majority of the trichloroacetic acid-soluble radioactivity (approximately 85%) is in the form of ¹²⁵I-moniodotyrosine. The extent and rapidity of cellular degradation of toxin suggest lysosomal proteolysis.

Effect of Concanavalin A on Toxin Degradation—The effect of concanavalin A (Con A) on the generation of trichloroacetic acid-soluble radioactivity is shown in Fig. 6. Con A was recently shown to inhibit the cellular internalization of diphtheria toxin without affecting toxin-receptor binding (13). In this system, it was shown that the Con A-induced inhibition of toxin internalization is accompanied by a block of the release of trichloroacetic acid-soluble radioactivity. By 180 min, nearly 60% of the original control cell-associated radioactivity was found in the form of trichloroacetic acid-soluble fragments in the culture medium, while less than 25% was degraded to trichloroacetic acid-soluble form by the cells treated with Con A. This demonstrates that receptor-mediated internalization is a prerequisite for degradation.

Effect of Specific Antibody on Internalization and Degradation—Internalization and degradation of diphtheria toxin by cells treated with antibody specific for diphtheria toxoid

FIG. 6. Effect of concanavalin A on toxin degradation. ¹²⁵I-toxin (0.03 μg/ml) was added to cells for 12 h at 4°C in the presence or absence of Con A (100 μg/ml). Cells were rinsed three times with HBSS, complete Hanks’ 199 with or without Con A (100 μg/ml) was added, and incubation was continued at 37°C. At the times indicated, triplicate samples were processed as described under ‘Experimental Procedures.’ TCA-soluble radioactivity: ○, control; □, Con A.
**Internalization and Degradation of Diphtheria Toxin**

**Effect of antibody on internalization and degradation of diphtheria toxin by Vero cells**

Cells were bound for 12 h at 4°C with 0.03 μg/ml of 125I-toxin. The monolayers were then rinsed three times with HBSS and incubated a further 30 min at 4°C in the presence or absence of 1:100 specific antibody. Cells were rinsed two times with HBSS, fresh complete medium was added, and cells were transferred to 37°C for the times listed below. Internalization and degradation were assayed in triplicate samples as described under “Experimental Procedures.” Standard error of the mean was in all cases less than 5%.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIHP-releasable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8,260</td>
<td>2,060</td>
</tr>
<tr>
<td>+ Antibody</td>
<td>7,960</td>
<td>3,350</td>
</tr>
<tr>
<td>PIHP-resistant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>560</td>
<td>2,210</td>
</tr>
<tr>
<td>+ Antibody</td>
<td>1,620</td>
<td>5,220</td>
</tr>
<tr>
<td>Trichloroacetic acid-soluble</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>4,580</td>
</tr>
<tr>
<td>+ Antibody</td>
<td>10</td>
<td>1,820</td>
</tr>
</tbody>
</table>

*Incubation time.*

was studied and the results (Table II) were analogous to those obtained with Con A. Here the cells were prebound with radiolabeled diphtheria toxin for 12 h at 4°C, rinsed, and incubated with specific antibody for 30 min. Following this incubation, cells were rinsed again, fresh medium was added, and the monolayers were transferred to 37°C. At various times after transfer, samples were harvested and assayed for toxin internalization and degradation.

Antibody at this concentration reproducibly interfered somewhat with the efficiency of the PIHP assay, although initial levels of total cell-associated radioactivity as calculated from the sum of PIHP-releasable and PIHP-resistant counts were essentially the same (control, 8,890 cpm; antibody-treated, 8,980 cpm). PIHP treatment removed only 82% of the receptor-bound toxin from the antibody-treated cells, as opposed to 94% from the controls. Therefore, in the antibody-treated samples the extent of internalization is probably overestimated. In experiments of this type it was found that internalization was 35 to 45% retarded in the antibody-treated cells relative to the controls, as determined by comparisons of the amount of PIHP-releasable radioactivity remaining after a 90-min incubation at 37°C. Concomitantly, toxin degradation, determined by measurement of trichloroacetic acid-soluble radioactivity in the culture medium, was 60 to 70% inhibited by antibody. This inhibition of degradation led to an accumulation of intracellular radioactivity; after a 90-min incubation at 37°C, there were over 2-fold more PIHP-resistant counts associated with the antibody-treated cells than with the controls.

These data strongly imply that cellular internalization of toxin is required for degradation. Also, since toxin-specific antibody (14) and Con A (15) effectively block the cytotoxic effects of diphtheria toxin at the concentrations used in these experiments (data not shown), the data support the hypothesis that diphtheria intoxication of mammalian cells requires sequential receptor binding, internalization, and intracellular processing and degradation.

**DISCUSSION**

The studies presented demonstrate that receptor-bound diphtheria toxin is rapidly internalized and degraded by sensitive mammalian cells. Since our previous work (1) showed that binding of toxin to specific receptor is a necessary step in diphtheria intoxication, it appears likely that the internalization process described here is responsible for the delivery of activated toxin (or toxin fragment) to the cytoplasm.

Certain other studies of diphtheria toxin uptake have indicated that both sensitive and resistant cells adsorb and degrade toxin by an apparently nonspecific pinocytotic mechanism (16, 17). It was hypothesized that this nonspecific process accounts for the vast majority of cellular toxin uptake, while a second undefined mechanism of entry accounts for the small number of toxin molecules capable of exerting the biological (cytotoxic) effect. A number of proposals have been put forward in attempts to define this second (specific) mechanism (17-19); most postulate some form of direct traversal of the plasma membrane by the toxin molecule (or a fragment).

Our system, while certainly not ruling out the possibility of multiple mechanisms of toxin entry, measures only uptake mediated by toxin-specific cell-surface receptors. The fate of receptor-bound toxin was studied initially using a technique which effectively differentiates between surface-bound and internalized toxin. At 4°C, under conditions in which endocytic uptake is minimal, over 85% of the specific cell-associated 125I-toxin was releasable by treatment with (PIHP) (Table I; Figs. 1 and 2). With increasing periods of incubation at 37°C, however, the cell-associated radioactivity became increasingly resistant to PIHP release, presumably due to progressive toxin internalization (Fig. 2). This susceptibility of cell surface-bound 125I-labeled diphtheria toxin to PIHP treatment is analogous to the release of surface-bound 125I-labeled EGF by proteolytic treatment with 0.25% trypsin (9). At 37°C, 125I-EGF was shown to become increasingly resistant to proteolytic release, suggesting progressive cellular internalization. Continued incubation at 37°C in the presence of 125I-EGF results in a loss of total radioactivity from the cells. These data were used by Carpenter and Cohen (6) to support the hypothesis that surface-bound EGF is sequentially internalized, degraded, and excreted by mammalian cells.

The value of biochemical techniques capable of releasing receptor-bound macromolecules from the cell surface is further exemplified by uptake studies utilizing the effect of sulfated glycosaminoglycans on cell-associated LDL (16). Heparin has been shown to elicit the dissociation of 125I-LDL from its cell surface receptor in a manner analogous to the release of receptor-bound concanavalin A by α-methyl-d-mannopyranoside. Heparin-mediated release was attributed to negative charges on the heparin molecule, which may interact with positively charged regions on LDL to form soluble complexes. A similar mechanism may account for the partial release of cell surface-bound diphtheria toxin by the polyanion inositol hexaphosphate. At 10 mg/ml, IHP was observed to effectively block the binding of 125I-labeled diphtheria toxin to its surface receptor. When cells were prebound at 4°C with 125I-toxin, a subsequent 60-min incubation with IHP induced the release of approximately half of the cell-associated radioactivity. Neither heparin nor dextran sulfate elicited 125I-labeled diphtheria toxin release, however, and IHP-mediated release was substantially enhanced by concomitant treatment with proteolytic enzyme, indicating that the observed detachment of surface-bound radioactivity is not solely attributable to receptor site competition by polyanion.

Receptor-mediated internalization of diphtheria toxin was accompanied by extensive degradation, as evidenced by the appearance of trichloroacetic acid-soluble radioactivity in the culture supernatants after periods of incubation at 37°C (Fig. 4). The trichloroacetic acid-soluble radioactivity was charac-

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Internalization and Degradation of Diphtheria Toxin

characterized by thin layer chromatography and found to consist primarily of 125I-monoiodotyrosine (Fig. 5). The rapidity and extent of the degradation process strongly imply the involvement of lysosomes. Since 60 to 70% of the cell-associated 125I-toxin is degraded in this manner, it appears that the majority of the cell-associated toxin is internalized, processed in lysosomes, and finally excreted in the form of single amino acids. This does not, however, preclude the possibility that a biologically active toxin fragment escapes lysosomal proteolysis. There is ample precedent in the literature for proteins which resist lysosomal digestion (20, 21); it is conceivable that such is the case with Fragment A. A lysosomal processing or degradation step has been indicated in the cellular uptake of numerous polypeptide hormones, growth factors, and essential serum macromolecules, including human choriogonadotropin (5), LDL (7), EGF (6), and α2-macroglobulin (22); it appears that the majority of the cell-associated diphtheria toxin may undergo a similar processing or degradation step. Additional support for this hypothesis is derived from the finding that the cytotoxic effects of diphtheria toxin are blocked by certain lysosomotropic agents, notably chloroquine.3

The apparent close coupling of internalization with degradation in our system makes it unlikely that we are merely observing penetration of the membrane by toxin. It seems more consistent with our results to suggest that internalization occurs by adsorptive endocytosis, followed by release of Fragment A from whole toxin in lysosomes. The internalization and degradation of receptor-bound diphtheria toxin appear to be directly related to expression of biological effect. When internalization and degradation are blocked by Con A (Fig. 6) or specific antibody (Table II), cytotoxicity is inhibited. We propose that, like certain polypeptide hormones, growth factors, and serum macromolecules, diphtheria toxin exerts its biological activity by sequential surface receptor binding, internalization via adsorptive endocytosis, and intracellular processing or degradation, probably in lysosomes.

3 S. H. Leppla, R. B. Dorland, and J. L. Middlebrook. manuscript submitted for publication.

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Receptor-mediated internalization and degradation of diphtheria toxin by monkey kidney cells.
R B Dorland, J L Middlebrook and S H Leppla


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