Inhibition of Coated Pit Formation in Hep_2 Cells Blocks the Cytotoxicity of Diphtheria Toxin But Not That of Ricin Toxin

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ABSTRACT It has been recently shown (Larkin, J. M., M. S. Brown, J. L. Goldstein, and R. G. W. Anderson, 1983, *Cell*, 33:273–285) that after a hypotonic shock followed by incubation in a K^+-free medium, human fibroblasts arrest their coated pit formation and therefore arrest receptor-mediated endocytosis of low density lipoprotein. We have used this technique to study the endocytosis of transferrin, diphtheria toxin, and ricin toxin by three cell lines (Vero, Wi38/SV40, and Hep_2 cells). Only Hep_2 cells totally arrested internalization of [^{125}I]transferrin, a ligand transported by coated pits and coated vesicles, after intracellular K^+ depletion. Immunofluorescence studies using anti-clathrin antibodies showed that clathrin associated with the plasma membrane disappeared in Hep_2 cells when the level of intracellular K^+ was low. In the absence of functional coated pits, diphtheria toxin was unable to intoxicate Hep_2 cells but the activity of ricin toxin was unaffected by this treatment. By measuring the rate of internalization of [^{125}I]ricin toxin by Hep_2 cells, with and without functional coated pits, we have shown that this labeled ligand was transported in both cases inside the cells. Hep_2 cells with active coated pits internalized twice as much [^{125}I]ricin toxin as Hep_2 cells without coated pits. Entry of ricin toxin inside the cells was a slow process (8% of the bound toxin per 10 min at 37°C) when compared to transferrin internalization (50% of the bound transferrin per 10 min at 37°C). Using the indirect immunofluorescence technique on permeabilized cells, we have shown that Hep_2 cells depleted in intracellular K^+ accumulated ricin toxin in compartments that were predominantly localized around the cell nucleus.

Our study indicates that in addition to the pathway of coated pits and coated vesicles used by diphtheria toxin and transferrin, another system of endocytosis for receptor-bound molecules takes place at the level of the cell membrane and is used by ricin toxin to enter the cytosol.

At present, the process by which macromolecules or macromolecular fragments enter the cell cytosol elicits considerable attention. Much of our knowledge concerning this process is based on the work on low density lipoprotein (LDL)[1] (1), Semliki forest virus (2), and diphtheria toxin (3). Molecules or viruses first bind specific receptors localized on the cell surface and are thereafter concentrated in specialized membrane domains called coated pits (4). They are then internalized by endocytosis into newly identified compartments (endosomes) (5) which may eventually fuse with lysosomes or elsewhere. It has been demonstrated that the endosomal compartment is rapidly acidified presumably by an active pumping of protons (6, 7). The low pH encountered in the endosome is used differently by macromolecules which enter into this particular cell compartment. Indeed some proteins dissociate from their receptors (such as LDL or asialoglycoproteins) (1, 8). Another remains firmly bound but loses a ligand (iron in the case of transferrin) (9). Certain viruses, such as

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; LDL, low density lipoprotein.

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Semliki forest virus, take advantage of the acidic pH to fuse their lipid envelope with the endosomal membrane, a process which leads to the transfer of their nucleocapsids into the cytosol (10). Diphtheria toxin uses also the endosomal low pH to create a "pore" with a hydrophobic portion of its B chain (11, 12) through which the catalytic A fragment of the toxin reaches the cytosol (13).

It has been recently shown by Larkin et al. (14) that after a hypotonic shock followed by incubation in a K^+-free medium, human fibroblasts arrest their coated pit formation and therefore arrest receptor-mediated endocytosis of LDL. We have used this protocol to study the entry into the cell cytosol of diphtheria and ricin toxins. These two proteins, or at least their catalytic subunits, have to cross the plasma membrane to exert their lethal activity (15). However, it has become clear that entry of these molecules into the cytosol is carried out by different mechanisms (16). One important question concerning these mechanisms was to determine whether both diphtheria and ricin toxins are necessarily taken up from the external medium by the same way of endocytosis (coated pits and coated vesicles) or if they use two different routes; namely coated pits, coated vesicles, endosomes for diphtheria toxin as evidenced by Keen et al. (17), and another kind of membrane retrieval process for ricin toxin.

In the present work, we show that in one cell line (Hep_2), internalization of transferrin, a ligand well known to be taken up by coated pits (18, 19), was totally blocked after intracellular K^+ depletion. Under these conditions, Hep_2 cells became totally resistant to diphtheria toxin, but were still fully sensitive to ricin toxin. It appears therefore that diphtheria toxin enters cells by coated pits whereas another system of endocytosis is required by ricin toxin to reach the cytosol and exert its activity.

MATERIALS AND METHODS

Cells: Vero cells (African green monkey kidney cells), Wi38/SV40 cells (human fibroblasts transformed by the simian virus 40), and Hep_2 cells (human carcinoma cells) were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DME) (Gibco Europe Ltd., Paisley, Scotland) complemented with 10% newborn calf serum, penicillin, and streptomycin.

Toxins and Antitoxins: Diphtheria toxin was obtained from Institut Pasteur Production (Louviers, France) and further purified by DE52 ion exchange chromatography. Diphtheria toxin was ~90% nicked as evidenced by PAGE analysis. Ricin toxin was purified from Castor beans as described (20). Toxins were kept frozen in small aliquots at −80°C and thawed only for use. Horse anti-diphtheria toxin serum (3,900 flocculation units/ml) was obtained from Institut Pasteur Production (Louviers, France). Rabbit ricin toxin antiserum was kindly provided by Dr. Bizzini, Institut Pasteur, Paris. One ml of anti-ricin toxin neutralized 1 mg of ricin toxin. For immunocytochemical localization of the ricin toxin into cells, anti-ricin toxin IgG was purified by affinity chromatography on a ricin toxin column as described (21).

Protocol for Hypotonic Shock of Cells, Estimation of Intracellular K^+ Content, and Measurement of Receptor-mediated Endocytosis of [^{125}I]Transferrin: The method of Larkin et al. (14) was followed to deplete cells of K^+ ions. Cells were grown in 24-well Multiwell tissue culture plates (Falcon Labware, Oxnard, CA) for 48 h (when they reached confluency). The medium was discarded and each monolayer was washed twice with 0.5 ml of 50 mM sodium HEPES buffer, pH 7.4, containing 100 mM NaCl. The cells were hypotonically shocked for 5 min by incubation in 0.5 ml of DME/water (1:1), followed by incubation for the indicated time in isotonic K^+-free medium (50 mM sodium HEPES, pH 7.4, 100 mM NaCl, 1 mM $CaCl_2$, 5 μg/ml insulin, 20 μg/ml transferrin, and 2 ng/ml selenious acid). Intracellular K^+ was determined essentially as described by Larkin et al. (14). Cell monolayers were washed four times with 100 mM $MgCl_2$ (0.5 ml per wash) and air dried at room temperature for 1 h. Monolayers were solubilized in 0.5 ml of 100 mM NaOH and centrifuged in a table top microfuge at maximum speed for 5 min. Determination of K^+ by flame photometry was done in the supernatants. For each determination, eight monolayers from a 24-well Multiwell tissue culture plate were used.

Human transferrin (Calbiochem-Behring Corp., San Diego, CA) was saturated with iron just prior to iodination and iodinated as described (9) with Na-[^{125}I] (Amersham Corp., U.K.). Binding assays of [^{125}I]transferrin to cells were performed in 24-well Multiwell tissue culture plates containing 2×10^6 cells per well. Before the binding assay, cells were washed twice at 4°C in binding medium (protein-free DME containing 20 mM sodium HEPES buffer, pH 7.3) and then incubated in the same medium for 30 min at 37°C to dissociate any receptor-bound transferrin molecules. Binding experiments were carried out in duplicate at 4°C for 2 h. After several washes, cells were solubilized in 1 M NaOH and the radioactivity was determined in a γ counter. Nonspecific binding (usually <10% of the specific binding) was determined by performing the binding assay in the presence of a 200-fold excess of unlabeled transferrin, and was substracted from all data.

Internalization of transferrin was measured by treatment of cells with pronase as described (19). Cells grown in 35-mm petri dishes (Falcon Labware) were depleted of K^+ as described above and [^{125}I]transferrin (5 nM) was added to the K^+-free isotonic medium without unlabeled transferrin at different times for 10 min at 37°C. Cell monolayers were washed with balanced saline medium (pH 7.4) at 4°C, followed by a 1-h incubation at 4°C in 1 ml of binding medium that contained 0.3% pronase (Boehringer Mannheim Diagnostics, Inc., FRG). At the end of the incubation, cells were completely detached from the dishes by repeated pipetting and centrifuged for 1 min at maximum speed in a table top microfuge. The radioactivity in the supernatants and the pellets was then determined.

Protein Determination: Protein contents were assayed according to Lowry et al. (22) using bovine serum albumin as a standard.

Measurement of Cell Protein Synthesis Inhibition by Diphtheria and Ricin Toxins: Cells were cultivated on 24-well Multiwell tissue culture dishes for 48 h before experiments. To each well, 0.5 ml of DME was added. Toxins to be tested were added at the indicated concentration and experiments were performed as described under each figure. At the end of the experiments, the medium was removed and 0.5 ml of a leucine-free medium that contained 0.1 μCi of [^{14}C]leucine (Centre de l'Energie Atomique, Saclay, France) was added in each well for 30 min. This medium was then removed and 1 ml of 10% TCA was added to each well. After 10 min at room temperature, the precipitated monolayers were washed carefully three times with 10% TCA (0.5 ml), and 0.5 ml of 0.1 M NaOH was added into each well to dissolve the cell monolayers. Each solubilized monolayer was then transferred into counting vials that already contained 4 ml of Instagel (Packard Instrument Co., Inc., Downers Grove, IL). The radioactivity was counted in a β counter. The results are expressed as a percentage of the incorporation in control samples (samples not treated with toxins).

Binding and Internalization of [^{125}I]Ricin Toxin by Hep_2 Cells: Ricin toxin was iodinated as described by Sandvig et al. (23). A specific activity of 10^5 cpm per μg of protein was used both for binding and internalization experiments. Binding studies were performed at 4°C using the same method described for the binding of [^{125}I]transferrin. Internalization of [^{125}I]ricin toxin was studied according to the method of Ray and Wu (24). Hep_2 cells cultivated in 35-mm petri dishes were depleted in intracellular K^+ and [^{125}I]ricin toxin was added at 5×10^{-8} M for the indicated periods of time. Cell monolayers were then rinsed with medium and incubated in balanced salt solution that contained 0.1 M lactose and 0.3% pronase for 1 h at 4°C. At the end of incubation, cells were completely detached from the dishes by repeated pipetting and centrifuged for 1 min in a table top microfuge at maximum speed. Radioactivity in the supernatants and the pellets was then determined.

Indirect Immunofluorescence Studies: After 48 h of growth on coverslips, cells were subjected to hypotonic shock, transferred in K^+ medium (with or without ricin toxin) as described above, and processed for localization of clathrin or ricin toxin by indirect immunofluorescence (25). The cells were fixed for 20 min at room temperature in 10 mM sodium phosphate buffer, pH 7.4, containing 3% (vol/vol) paraformaldehyde, 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$, and then washed twice with 10 mM sodium phosphate buffer, pH 7.4. Each monolayer was then permeabilized with 2 ml of 0.1% (vol/vol) Triton X-100 in 10 mM sodium phosphate buffer, pH 7.4. Each coverslip was then placed in a petri dish and covered by a 100-μl drop of diluted affinity-purified rabbit anti-clathrin (10 μg/ml) or rabbit anti-ricin toxin IgG, and incubated for 20 min at room temperature. After four 15-min washes with 10 mM sodium phosphate buffer, pH 7.4, that contained 0.2% gelatin, the cells were incubated with 100 μl of diluted goat anti-rabbit IgG conjugated either with fluorescein or rhodamine (Nordic, Tilburg, Netherlands) at room temperature for 20 min. The coverslips were washed and mounted in glass slides, examined, and photographed under a fluorescence microscope.

Preparation of the Fluorescein Isothiocyanate–labeled

Transferrin: Human transferrin was loaded with iron and extensively dialyzed against phosphate balanced salt solution. The final concentration of ferrotransferrin was 1.12 mg/ml. A solution of fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, MO) at 10 mg/ml in 1 M $NaCO_3H$ was prepared just prior to addition to transferrin. To 1 mg of ferrotransferrin, either 20 or 28 μg of FITC was added in 60 mM Na_2CO_3, pH 9.25. The mixture was incubated for 1 h at room temperature in the dark. FITC-labeled transferrin was separated from free FITC by gel filtration on a G25 column, equilibrated with 20 mM sodium HEPES buffer, pH 7.2, containing 150 mM NaCl. FITC-labeled transferrin was shown to bind specifically to its receptors since in the presence of a 100-fold excess of free ferrotransferrin, no label was observed on cell preparations.

RESULTS

Intracellular K^+ Depletion Totally Blocked Internalization of [^{125}I]Transferrin in Hep_2 Cells

Three different cell lines were tested for their ability to arrest internalization of proteins mediated by coated pits after intracellular K^+ depletion. Transferrin was used as a probe to test this particular pathway since it is well documented that receptors for this protein are present in all cell lines and endocytosed in coated pits. We first checked that iodinated transferrin was able to bind cells specifically, and the number of transferrin receptors present in Vero, Wi38/SV40, and Hep_2 cells was estimated. As shown in Fig. 1, *A* and *C*, Vero and Wi38/SV40 cells had 90,000 and 300,000 receptors per cell respectively. About 10^6 receptors for transferrin were found in Hep_2 cells (Fig. 1 *B*). A high number of transferrin binding sites has already been reported for HeLa cells (26) which are very similar to Hep_2 cells. Only one class of transferrin receptors was found on Hep_2 cells, with an apparent affinity constant of 50 nM as shown by Scatchard analysis (Fig. 1 *D*). This is close to the value of 27 nm found by Ward et al. in the case of HeLa cells (26). When the binding assay was performed after hypotonic shock and in the absence of K^+, no modification of transferrin binding could be found (Fig. 2).

When Vero, Wi38/SV40, or Hep_2 cells were subjected to hypotonic shock and then transferred into isotonic K^+-free medium, the cellular K^+ level declined rapidly for the three cell lines during the first 15 min (Fig. 3). Intracellular K^+ levels for Wi38/SV40 and Hep_2 cells then remained constant for up to 60 min at a value of 40% for Wi38/SV40 and 25% of K^+-containing Hep_2 cells (Fig. 3). The intracellular K^+ level of Vero cells declined slightly from 15 to 60 min to reach a value of 30% of control. It can be noticed from Fig. 3 that Hep_2 cells reached the lowest value of intracellular K^+ level more rapidly.

Ledbetter and Lubin (27) have shown that K^+ depletion reduced protein synthesis in cultured cells without affecting the rate of their amino acid uptake. Since the aim of our work was to study the activity of toxins which inhibit protein synthesis on K^+-deprived cells, we have checked that, after the hypotonic shock followed by incubation in K^+-free medium, subsequent addition of K^+-containing culture medium did restore protein synthesis. Vero, Wi38/SV40, or Hep_2 cells were therefore depleted in intracellular K^+, and after 45 min in K^+-free buffer, they were washed and incubated for 24 h in culture medium that contained potassium. Their protein

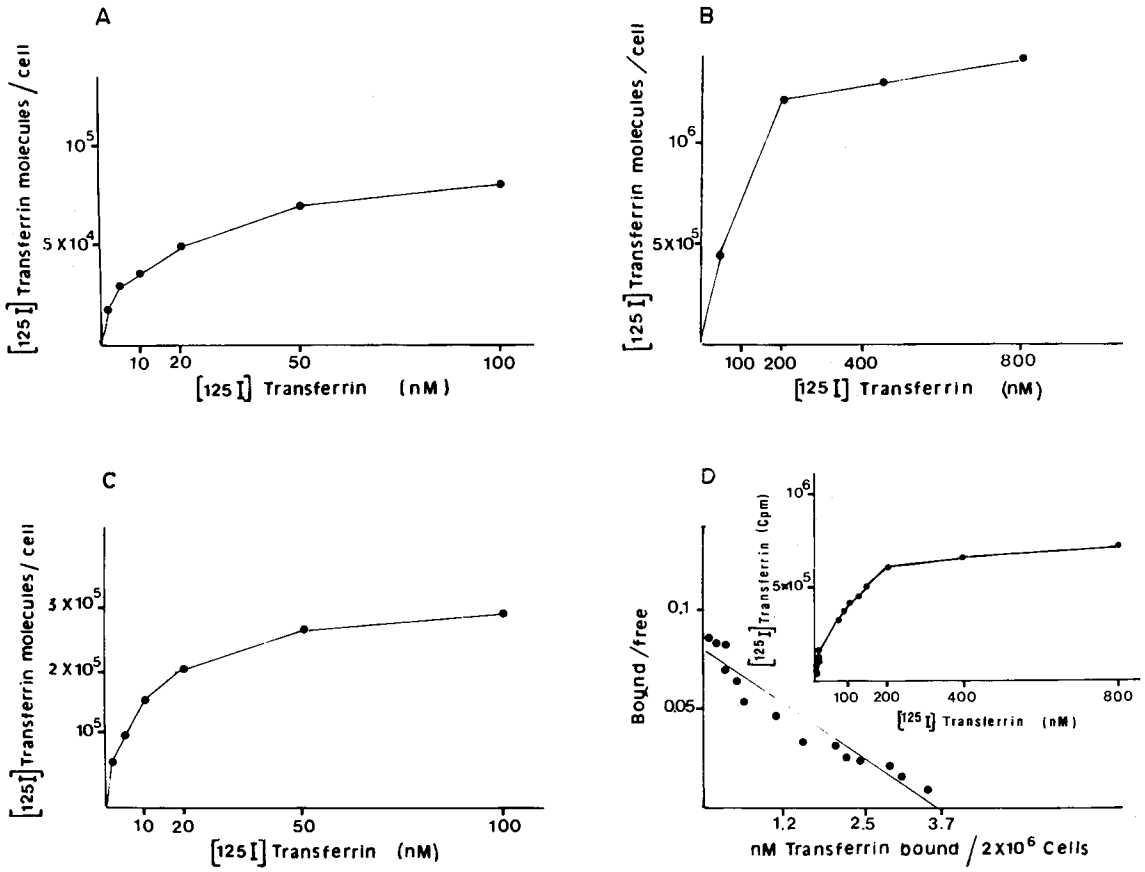

FIGURE 1 Binding of [^{125}I]transferrin at 4°C to Vero (*A*), Hep_2 (*B*), and Wi38/SV40 (*C*) cells. Binding experiments were performed in duplicate as described in Materials and Methods. Nonspecific counts (<10% of specific counts) were subtracted from all data. Scatchard analysis of a binding experiment of [^{125}I]transferrin to Hep_2 cells at 4°C is given in *D*.

synthesis was then measured by incorporation of radiolabeled leucine. We found that in these conditions cells did restore 80% of their protein synthesis (data not shown). It was thus decided that after 15 min of K^+ depletion, toxins would be added to cells for 30 min. Therefore, the time during which cells would be depleted in intracellular K^+ would never exceed 45 min.

The rate of internalization of [^{125}I]transferrin in Vero, Wi38/SV40, and Hep_2 cells was studied by following the rate of pronase resistance of the radiolabeled ligand at 37°C (20). After hypotonic shock and various times (5, 20, 35, 40, and 65 min) in K^+-free isotonic buffer, [^{125}I]transferrin was added to cells for 10 min at 37°C (Fig. 4). After these pulses of [^{125}I]transferrin, cell monolayers were processed for pronase treatment as described in Materials and Methods.

From Fig. 4*A* it can be seen that both K^+-containing and K^+-depleted Vero cells internalized [^{125}I]transferrin. Indeed half of the ligand was found associated with the cells whereas half of it was digested by pronase (Fig. 4*A*). In the case of Wi38/SV40 cells, after a 35-min incubation in K^+-free buffer, a slight decrease in transferrin internalization could be observed when compared to control cells in K^+-containing medium (Fig. 4*B*). In contrast, internalization of [^{125}I]transferrin was totally blocked in Hep_2 cells when the intracellular K^+ was low (Fig. 4*C*). Even a 5-min incubation in K^+-free medium after hypotonic shock totally stopped the entry of [^{125}I]transferrin into the cells (Fig. 4*C*). In the presence of K^+, Hep_2 cells internalized transferrin at the same rate as Vero or Wi38/SV40 cells (50% sensitive to pronase, 50% resistant after 10 min at 37°C [Fig. 4*C*]). It can be inferred from these experiments that, after hypotonic shock, Hep_2 cells rapidly

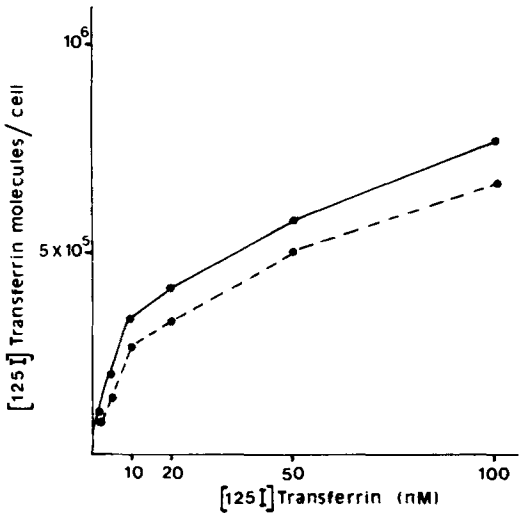

FIGURE 2 Binding of [^{125}I]transferrin at 4°C to Hep_2 cells before intracellular K^+ depletion (-----) and after intracellular K^+ depletion (—). Cells were depleted of K^+ as described in Materials and Methods, incubated in isotonic K^+-free medium at 4°C, and binding experiments were performed as in Fig. 1.

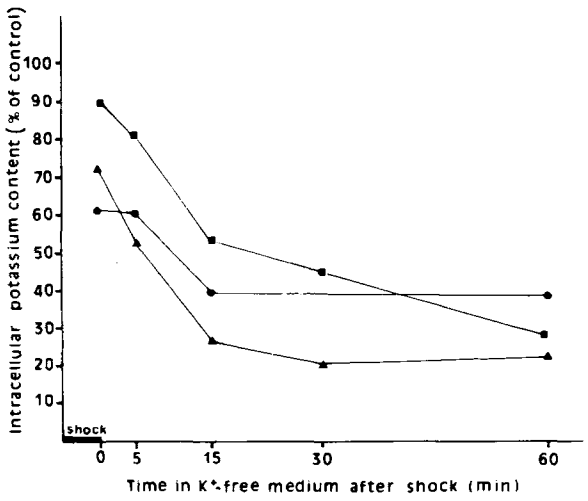

FIGURE 3 Decline in intracellular K^+ concentration in Vero (■), Wi38/SV40 (●), and Hep_2 (▲) cells after hypotonic shock in K^+-free medium. Cells were processed for hypotonic shock and incubated in K^+-free medium for the indicated periods of time after shock. Concentration of intracellular K^+ was estimated by flame photometry. Results are expressed as % of control cells not depleted in K^+.

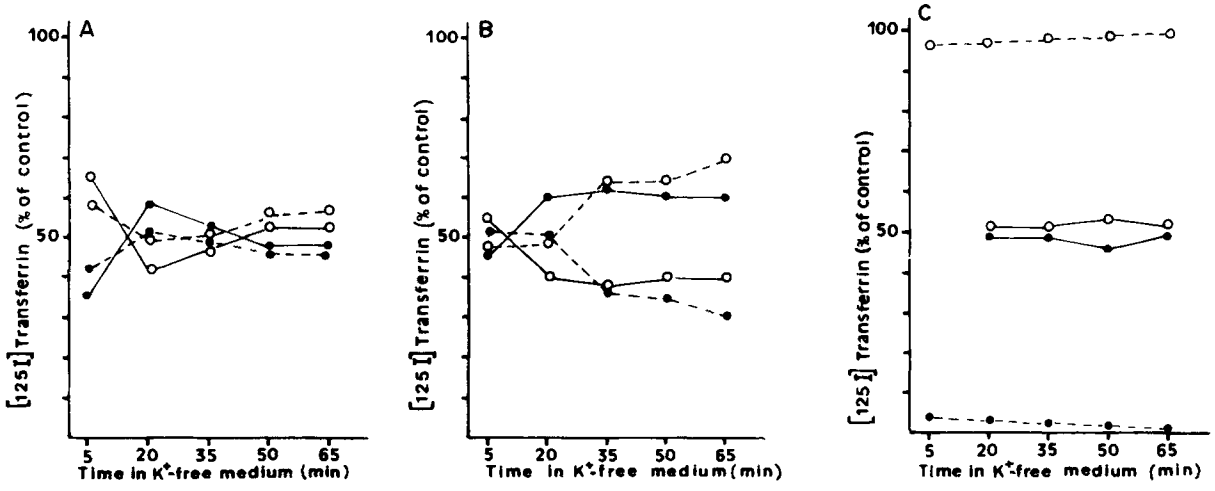

FIGURE 4 Internalization of [^{125}I]transferrin by Vero (*A*), Wi38/SV40 (*B*), and Hep_2 (C) cells after intracellular K^+depletion (-----) or with normal K^+ content (—). Cells for experiments performed with a low intracellular K^+ were processed as described in Materials and Methods. Labeled transferrin was added to cells for 10-min pulses at 37°C at the indicated times after hypotonic shock. Non-internalized ligand was digested by pronase as described in Materials and Methods. After pronase treatment, cell monolayers were detached from the petri dishes, centrifuged, and radioactivity in the pellet (●) (representing the pronase-resistant fraction) and in the supernatant (○) (representing the pronase-sensitive fraction) was counted.

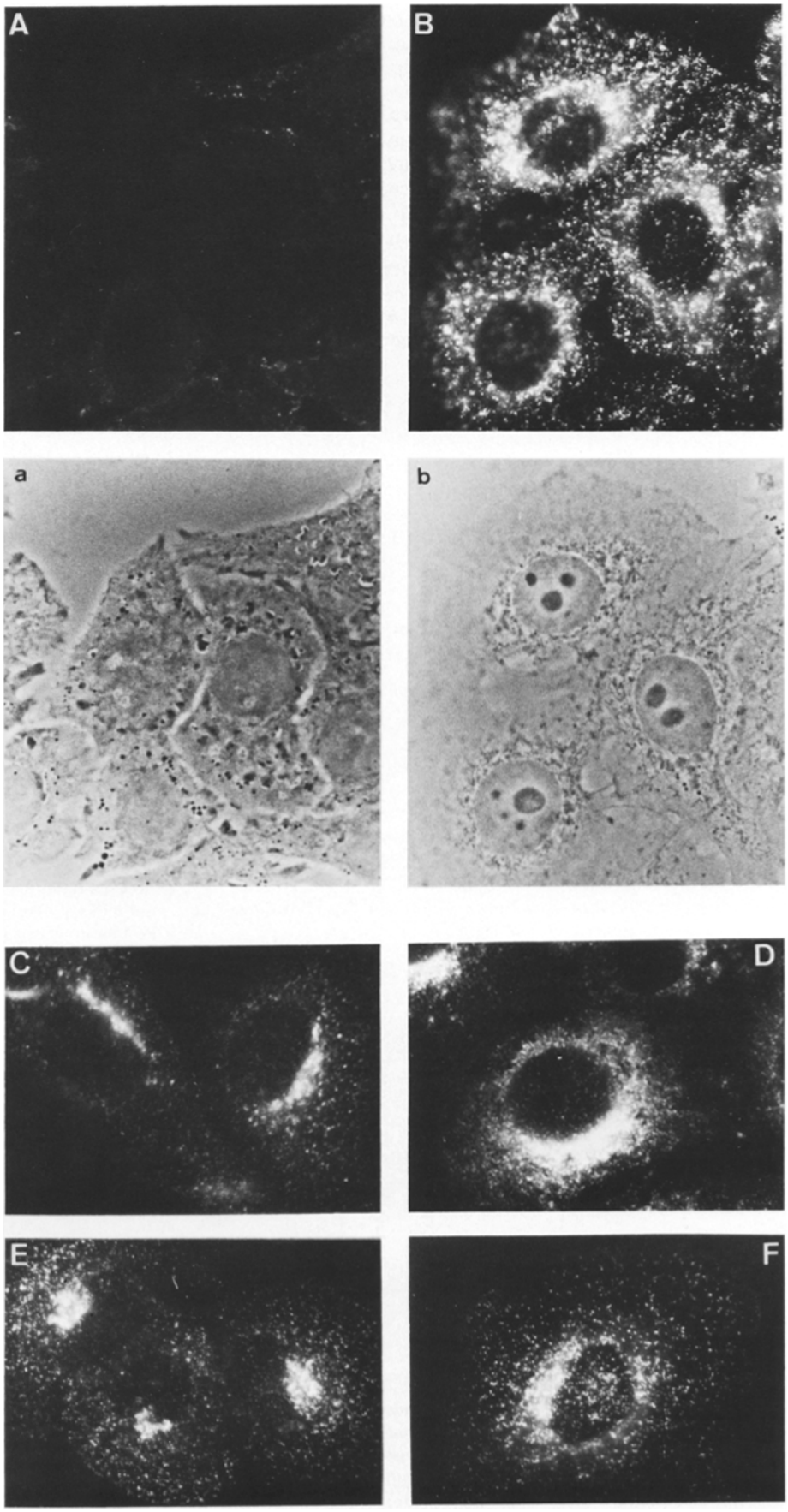

FIGURE 5 Localization of intracellular clathrin, by indirect immunofluorescence, in intracellular K^+-depleted cells or in cells with a normal K^+ content. Cells cultivated on coverslips were subjected to hypotonic shock and transferred into isotonic K^+-free medium for 15 min at 37°C. Cells were then fixed, permeabilized, and processed for indirect immunofluorescence as described in Materials and Methods. (*A*, *C*, and *E*) K^+-depleted cells Hep_2, Vero, and Wi38/SV40, respectively. (*B*, *D*, and *F*) K^+-containing cells (Hep_2, Vero, and Wi38/SV40, respectively). Parallel phase-contrast micrographs of Hep_2 cells are shown in *a* and *b*.

arrest internalization of [^{125}I]transferrin whereas Vero or Wi38/SV40 cells do not.

Clathrin Associated With the Surface Membrane of Hep$_2$ Cells Disappeared After Intracellular K$^+$ Depletion

Localization of clathrin in Hep$_2$, Vero, and Wi38/SV40 cells was determined by an indirect immunofluorescence technique (25) using rabbit antibodies directed against clathrin (heavy and light chains) (28). As shown in Fig. 5, *B*, *D*, and *F*, in the presence of K$^+$, clathrin is found in two distinct cell localizations. A peripheral localization at the level of the cell membrane is shown by the presence of small dots representing clathrin that is associated with coated pits or coated vesicles. A second set of clathrin localization was detected around the nucleus and represents clathrin that is associated with the Golgi apparatus.

After shock and transfer of cells in K$^+$-free medium, the typical punctuate pattern of clathrin was not observed in Hep$_2$ (Fig. 5*A*) but was still present in Vero and Wi38/SV40 cells (Fig. 5, *C* and *E*). It appears therefore that only in Hep$_2$ cells, intracellular K$^+$ depletion induced the total dissociation of clathrin from cell membranes and consequently coated pit formation. It has been already shown that the antibodies directed against clathrin, used in this experiment, do not detect unassembled clathrin in cells (28).

Hep$_2$ Cells Became Totally Resistant to Diphtheria Toxin But Remained Fully Sensitive to Ricin Toxin After Intracellular K$^+$ Depletion

Activities of ricin and diphtheria toxins on cells, after hypotonic shock and intracellular depletion were tested according to the following protocol. Cells were subjected to a hypotonic shock for 5 min and then transferred into an isotonic K$^+$-free medium for 15 min. Diphtheria or ricin toxin was thereafter added at various concentrations for 30 min at 37°C. Cells were then washed with K$^+$-free buffer that contained either 100 mM lactose to remove ricin toxin molecules bound on the cell surface (29) or 10 mM ammonium chloride to raise endosomal pH preventing the escape of diphtheria toxin from this compartment (30), and incubated in culture medium that contained 40 mM lactose or 10 mM NH_4Cl at 37°C. Lactose or ammonium chloride were shown to block 100% of ricin or diphtheria toxin activity by the following experiment. Cells at 4°C were incubated with either ricin or diphtheria toxin for 1 h. The monolayers were then washed at 4°C and culture medium that contained lactose (in the case of ricin toxin) or ammonium chloride (in the case of diphtheria toxin) was added. After an overnight incubation at 37°C, protein synthesis was estimated. Control experiments were carried out on cells not hypotonically shocked and not depleted of intracellular K$^+$. As shown in Fig. 6*A*, Hep$_2$ cells after shock and K$^+$ depletion became totally resistant to diphtheria toxin whereas only a small but clear protection was observed in Wi38/SV40 cells (Fig. 6*B*). No protection against diphtheria toxin was induced by lowering the intracellular potassium content in the case of Vero cells (Fig. 6*C*). For ricin toxin no protection was observed in any of the three cell lines tested after intracellular K$^+$ depletion (Fig. 7, *A–C*).

Yamaizumi et al. (31) have shown that a single molecule of diphtheria toxin fragment A left for 48 h in the cell cytoplasm is sufficient to totally arrest protein synthesis. To be certain that not even one molecule of diphtheria toxin

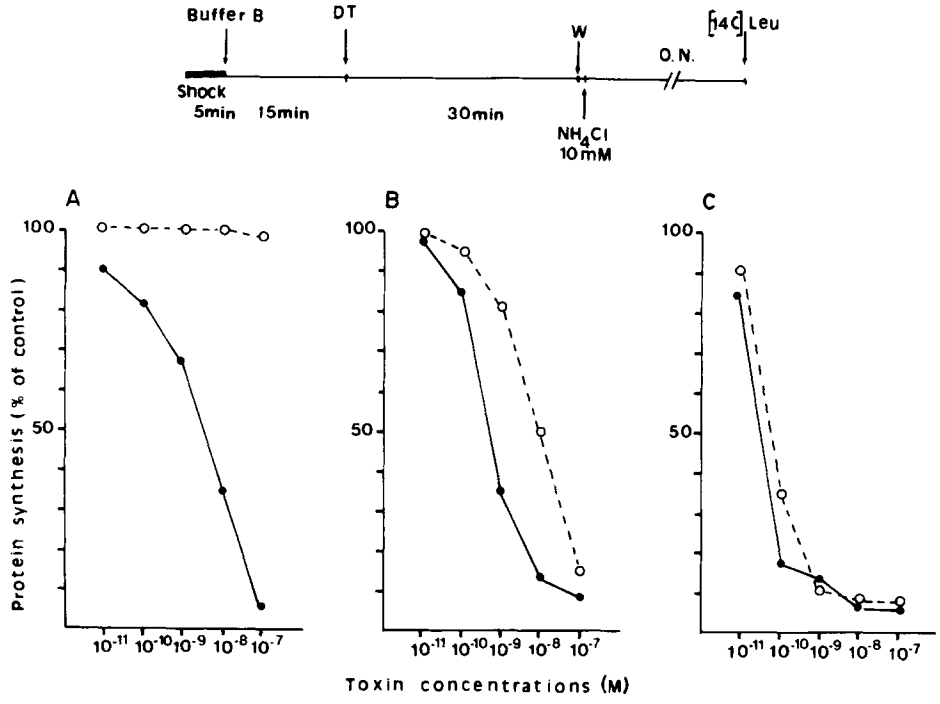

FIGURE 6 Effect of diphtheria toxin on protein synthesis of Hep$_2$ (*A*), Wi38/SV40 (*B*), and Vero (C) cells after intracellular K$^+$ depletion (○) or with a normal K$^+$ content (●). Experiments were conducted as shown in the schematic representation above the figure. After a 5-min hypotonic shock and 15-min incubation in isotonic K$^+$-free medium (buffer B), various concentrations of diphtheria toxin (*DT*) were added to the cells for 30 min at 37°C. Cells were then washed (*W*) three times with buffer B containing 10 mM NH_4Cl at 4°C and incubated overnight at 37°C in DME containing 10 mM NH_4Cl. Protein synthesis was then estimated by incorporation of [^{14}C]leucine ([^{14}C]*Leu*) as described in Materials and Methods. Protein synthesis is expressed as per cent of control cells (not treated by diphtheria toxin).

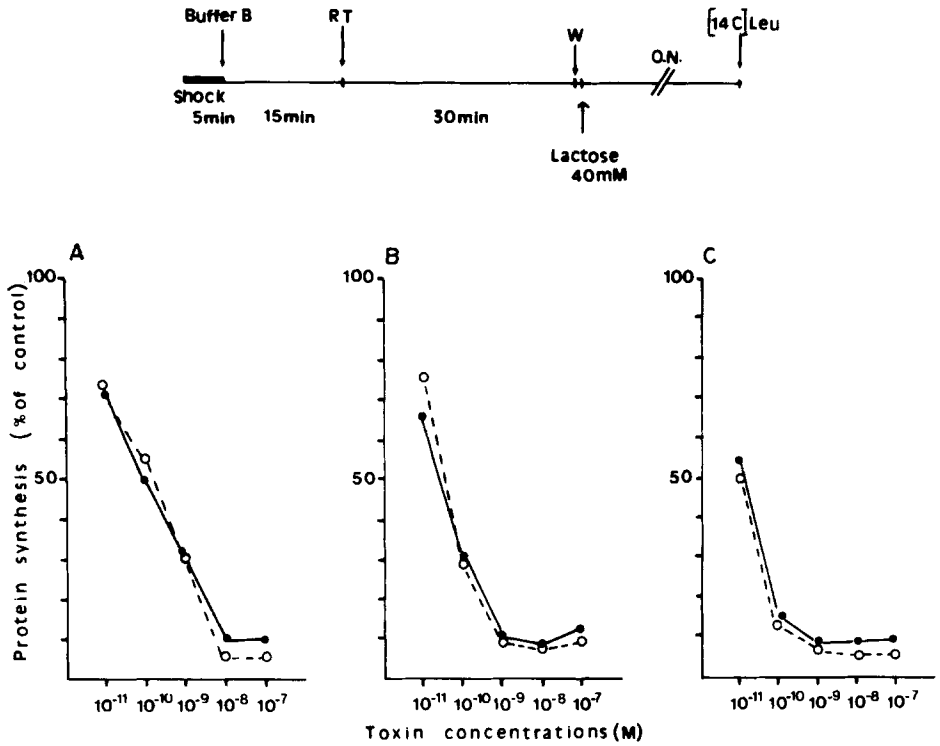

FIGURE 7 Effect of ricin toxin on protein synthesis of Hep_2 (*A*), Wi38/SV40 (*B*), and Vero (C) cells after intracellular K^+ depletion (○) or with a normal K^+ content (●). Experiments were conducted as schematically represented above the figure. After a hypotonic shock of 5 min, cells were transferred in isotonic K^+-free medium for 15 min, (buffer B); various concentrations of ricin toxin (*RT*) were then added to the cells for 30 min at 37°C. Cells were then washed (*W*) three times at 4°C with buffer B containing 40 mM lactose and incubated overnight at 37°C in DME containing 40 mM lactose. Protein synthesis was then assayed by incorporation of [^{14}C]leucine ([^{14}C]*Leu*) as described in Materials and Methods. Protein synthesis is expressed as percent of control cells (not treated with ricin toxin).

fragment A was transferred into the cytoplasm of K^+-depleted cells during the 30-min incubation period, we have measured protein synthesis after 48 h in the presence of anti-diphtheria toxin antibodies to block further entry of this molecule into cells (31). The same type of experiment was done with ricin toxin. It is clear from Fig. 8*A* that Hep_2 cells after intracellular K^+ depletion were totally protected against diphtheria toxin, whereas Vero cells in the same conditions were fully intoxicated (Fig. 8*B*). In contrast, no protection was afforded by intracellular K^+ depletion in the case of ricin toxin on both Hep_2 and Vero cells (Fig. 8*C* and 8*D*). To check that the hypotonic shock followed by incubation in isotonic K^+-free medium did not affect diphtheria toxin binding on Hep_2 cells, the following experiment was done. Hep_2 cells were hypotonically shocked for 5 min and then transferred in isotonic K^+-free medium for 15 min at 37°C and cooled down to 4°C to block the endocytosis process. Diphtheria toxin was then added for 30 min at 4°C. Under these conditions, only binding of toxin occurs. The cells were then washed to remove unbound toxin molecules and incubated overnight at 37°C in culture medium that contained K^+. Protein synthesis was then estimated. Control experiments with Hep_2 cells not depleted of K^+ were run in parallel. As shown in Table I, after intracellular K^+ depletion, inhibition of protein synthesis by diphtheria toxin was obtained at a level similar to that of cells having a normal K^+ content. This shows that after shock and in isotonic K^+-free medium, diphtheria toxin bound to Hep_2 cells normally and that no loss of toxin receptors occurred.

Internalization of [^{125}I]Ricin Toxin Was Reduced But Not Blocked by Intracellular K^+ Depletion of Hep_2 Cells

Binding and internalization of [^{125}I]ricin toxin was studied in K^+-containing and K^+-depleted Hep_2 cells. We have followed internalization of [^{125}I]ricin toxin during 10-min pulses to compare the entry, within a cell compartment, of this toxin with that of transferrin. As evidenced by Table II, in Hep_2 cells having a normal K^+ content, a very small amount of [^{125}I]ricin toxin entered during a 10-min pulse (~8% of the bound toxin). Table II shows that [^{125}I]ricin toxin molecules did not enter into Hep_2 cells merely by fluid phase pinocytosis since in the presence of lactose, which competes for toxin receptors, no labeled ricin toxin was internalized. This result agrees with a previous work of Sandvig et al. (32), who found that in HeLa cells ~3% of surface-bound ricin toxin was internalized (not releasable by lactose) during a 10-min incubation at 37°C. However, it differs slightly from that of Ray and Wu (24) who found that 25% of surface-bound [^{125}I]ricin toxin was internalized into Chinese hamster ovary cells during 10 min of incubation at 37°C. After shock and intracellular K^+ depletion, internalization of [^{125}I]ricin toxin was partially reduced (by <50%) (Table II). Binding of ricin toxin to K^+-containing and K^+-depleted Hep_2 cells was very similar (see Table II). It can be concluded, therefore, that when clathrin is dissociated from the cell surface, ricin toxin does enter Hep_2 cells although in a lesser amount. This process is slow (with

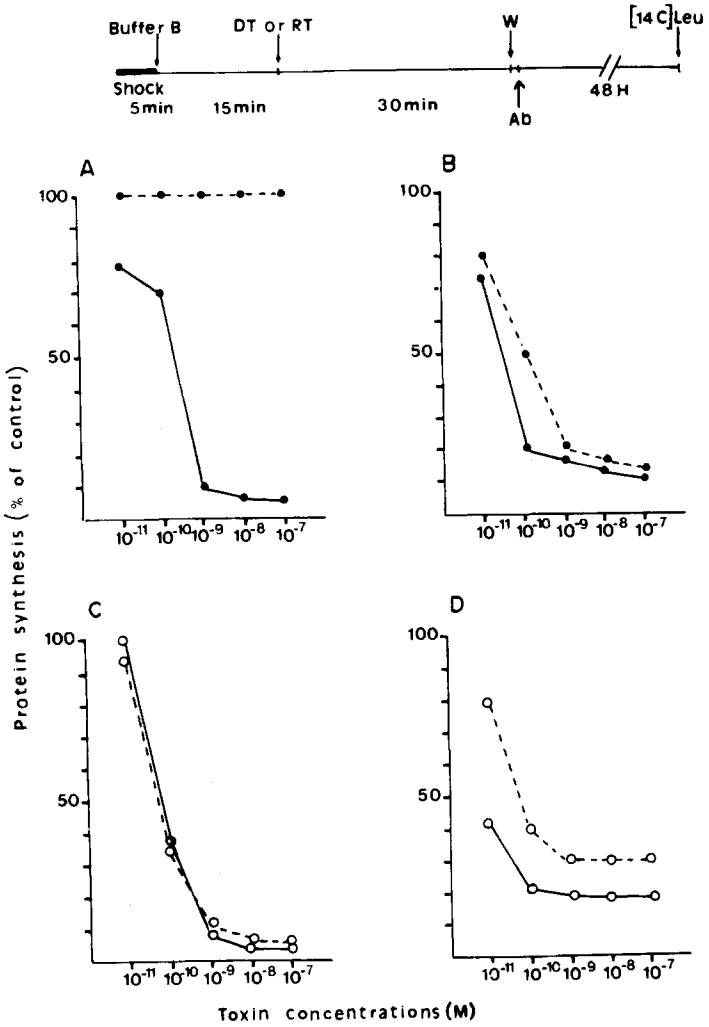

FIGURE 8 Effects of diphtheria toxin (*A* and *C*) and ricin toxin (*B* and *D*) on cell protein synthesis after a 48-h incubation at 37°C, on Hep_2 cells (●) or Vero cells (○) after intracellular K^+ depletion (-----) or with a normal K^+ content (—). Experiments were conducted as represented in the schematic drawing above the figure. After a 5-min hypotonic shock and incubation for 15 min in an isotonic K^+-free medium (buffer B), various concentrations of diphtheria or ricin toxin were added to the cells. After 30 min at 37°C, cells were carefully washed three times with buffer B containing either 20 flocculation units of horse anti-diphtheria toxin serum (anti-DT) or 10 μl of rabbit anti-ricin toxin serum (anti-RT) per ml buffer B. Cells were then incubated at 37°C for 48 h in DME containing, in the case of diphtheria toxin experiments, 20 flocculation units of horse anti-DT serum per ml, or, in the case of experiments performed with ricin toxin, 10 μl of rabbit anti-RT per ml. Protein synthesis was then assayed by incorporation of [^{14}C]leucine ([^{14}C]*Leu*) as described in Materials and Methods. Protein synthesis is expressed as percent of control cells (not treated with toxin). *Ab*, antibody added.

or without functional coated pits) when compared to that of transferrin in which 50% of the bound molecules were inside the cell after 10 min at 37°C (Fig. 4*C*).

Lysosomotropic drugs such as NH_4Cl, chloroquine, or methylamine have been shown to sensitize cells to abrin and ricin toxins (16, 33). Since these agents act by raising the pH of intracellular compartments, such as endosomes or lysosomes, we have tested the effects of NH_4Cl on the toxicity of ricin toxin using Hep_2 cells with coated pits in activity (Hep_2 cells in K^+-containing medium) or Hep_2 cells that are blocked (Hep_2 cells in isotonic K^+-free medium). As previously reported, sensitization to ricin toxin by NH_4Cl was observed on cells with active coated pits (Fig. 9). However, in Hep_2 cells unable to take up transferrin by coated vesicles, no sensitization to ricin toxin by NH_4Cl could be found (Fig. 9). This observation is compatible with the idea that ricin toxin is usually taken up by two systems of endocytosis: the acidic pathway of coated pits, coated vesicles, and endosomes which, in normal circumstances, probably do not deliver ricin toxin inside the cytosol; and another form of endocytosis, possibly in vesicles with neutral pH, which could be the route of this molecule to exert its lethal activity. Upon incubation with NH_4Cl, the acidic pathway is neutralized and ricin toxin (in addition to its normal route of entry) can penetrate the cytosol through it. Thus, a higher amount of ricin toxin might penetrate inside the cell successfully, resulting in an apparent increased toxicity of this protein.

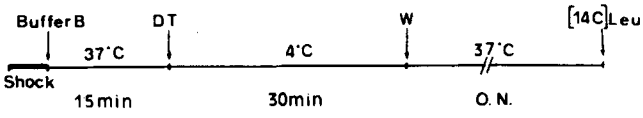

TABLE I. *Effect of Intracellular Potassium Depletion on the Binding of Diphtheria Toxin to Hep_2 Cells*

Intracellular K^+ content	Diphtheria toxin	Protein synthesis cpm	Protein synthesis inhibition
	M	*cpm*	*% of control*
+	none	9,915	5
+	10^{-8}	498	
−	none	8,123	5, 8
−	10^{-8}	473	

After a 5-min hypotonic shock, cells were incubated for 15 min at 37°C in isotonic K^+-free medium (buffer B); diphtheria toxin was then added at the indicated concentration (*DT*) for 30 min at 4°C. The cells were then washed (*W*) and incubated overnight at 37°C in DME (*O.N.*). Protein synthesis was then assayed as described in Materials and Methods. Control experiments were done with cells having a normal K^+ content with an identical protocol.

Morphological Studies

To determine the localization of ricin toxin in Hep_2 cells with and without functional coated pits, we have incubated Hep_2 cells (either deprived of K^+ or with normal K^+ content) with both FITC-labeled transferrin (to follow the internaliza-

TABLE II. *Internalization of [^{125}I]Ricin Toxin in K$^+$-containing and K$^+$-depleted Hep$_2$ Cells*

Intracellular K$^+$ content	Time of internalization after shock	[^{125}I]Ricin toxin bound (pronase sensitive)			[^{125}I]Ricin toxin internalized (pronase resistant)			[^{125}I]Ricin toxin internalized
		With 100 mM lactose A	Without lactose B	Specific counts B − A	With 100 mM lactose C	Without lactose D	Specific counts D − C	
	min	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*	*cpm*	*% bound*
+	5–15	2,045	25,485	23,440	112	1,964	1,852	8
−	5–15	2,024	30,724	28,700	137	1,259	1,122	4
+	15–25	2,061	32,154	30,093	267	2,499	2,232	7.4
−	15–25	2,192	44,166	41,974	174	2,418	2,244	5.3
+	25–35	1,737	29,600	27,863	201	2,441	2,440	8
−	25–35	2,011	30,817	28,806	138	1,623	1,485	5.2
+	35–45	1,628	22,192	20,564	194	1,847	1,653	8
−	35–45	1,558	24,340	22,782	104	884	780	3.4

Cells for experiments performed with a low intracellular K$^+$ content were hypotonically shocked and then incubated in isotonic K$^+$-free medium. Labeled ricin toxin was added to cells for 10-min pulses with or without 100 mM lactose at the indicated time after hypotonic shock. Non-internalized ricin toxin was digested by 0.5% pronase in the presence of 100 mM lactose at 4°C for 1 h (as indicated in Materials and Methods). After pronase treatment, cell monolayers were completely detached from the petri dishes, centrifuged, and the radioactivity in the pellet (representing the pronase-resistant fraction) and in the supernatant (representing the pronase-sensitive fraction) was counted. When Hep$_2$ cells were incubated at 0°C with [^{125}I]ricin toxin and then processed by pronase as described above, 0.8% of the bound radioactivity was found to remain associated with cells (data not shown). This value can be considered as the intrinsic background of the assay.

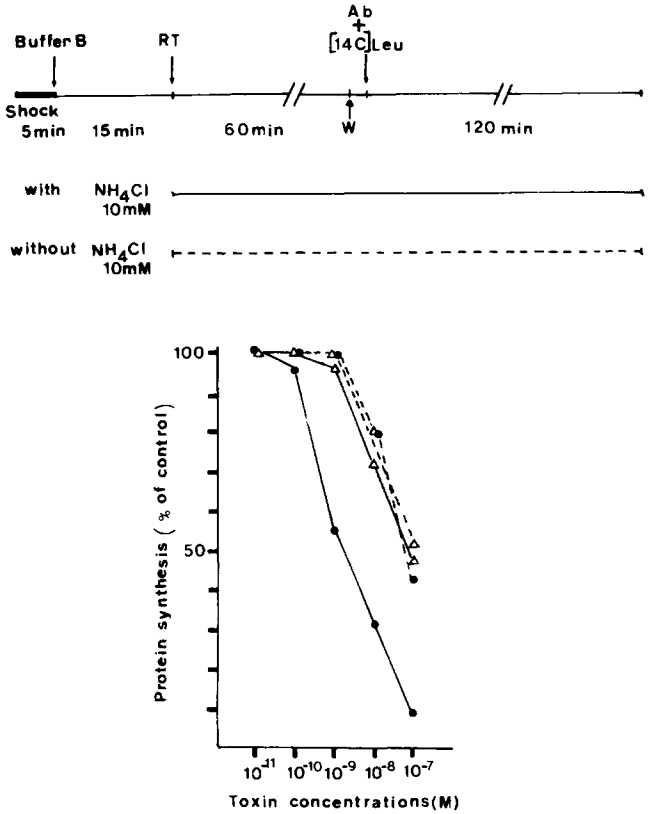

FIGURE 9 Effect of NH_4Cl on ricin toxin activity on Hep$_2$ cells with and without active coated pits. Experiments were conducted as represented in the schematic drawing above the figure. After a 5-min hypotonic shock, cells were incubated in isotonic K$^+$-free buffer (buffer B). Various concentrations of ricin toxin were added to the cells either in the presence of 10 mM NH_4Cl (—) or in the absence of it (-----). After 1 h at 37°C cells were washed three times with buffer B at 4°C and incubated at 37°C for 120 min in DME containing 10 μl of rabbit anti-ricin toxin serum per ml (*Ab*), 0.2 μCi of [^{14}C]leucine per ml, with or without 10 mM NH_4Cl. Cells were then processed for protein synthesis estimation as described in Materials and Methods. △, K$^+$-depleted cells; ●, nondepleted cells.

tion mechanism by coated pits) and ricin toxin. After incubation for 30 min at 37°C, cells were washed with cold medium and incubated with lactose at 4°C for 15 min to remove membrane-bound ricin toxin. Cells were then fixed, permeabilized with Triton X-100, and incubated with affinity-purified rabbit IgG directed against ricin toxin. Further incubation with rhodamine-labeled goat antibodies directed against rabbit IgG allowed identification of ricin toxin by fluorescence. As shown in Fig. 10*A*, in Hep$_2$ cells with a normal K$^+$ content, FITC-labeled transferrin was seen in the cell cytoplasm mainly as punctuate dots representing molecules either bound to surface receptors or in endosomal vesicles. No clear accumulation in a particular cell compartment could be observed. Ricin toxin in the same cells (Fig. 10*C*) was largely accumulated around the cell nucleus and particularly at one pole of it. Vesicles were seen containing both fluorescein- and rhodamine-labeled molecules. Experiments performed with Hep$_2$ cells in which internalization by coated pits was blocked by potassium starvation are seen in Fig. 10*B*, and *D–F*. FITC-labeled transferrin was seen as punctuate dots localized on the cells (Fig. 10*B*). The same pattern for FITC-labeled transferrin localization was obtained on Hep$_2$ cells not permeabilized by Triton X-100 (Fig. 10*E*). In these cells, ricin toxin was seen accumulated at the level of the cell nucleus and observed as dense patches (Fig. 10*D*) but having a somewhat different morphology from that of Hep$_2$ cells with active coated pits. When the cells incubated with ricin toxin were not permeabilized (Fig. 10*F*), no rhodamine fluorescence could be detected, indicating that ricin toxin, seen in Fig. 10*D*, was clearly inside the cell.

DISCUSSION

It was reported by Larkin et al. (14) that by lowering the intracellular K$^+$ level in fibroblasts, receptor-mediated endocytosis of LDL and coated pit formation could be inhibited. We have used the same method to lower the intracellular K$^+$ level in three cultured cell lines. In one cell line (Hep$_2$), where the decline in intracellular K$^+$ after hypotonic shock was the most rapid, receptor-mediated endocytosis of transferrin was totally arrested and association of clathrin with the plasma membrane disappeared. Endocytosis of transferrin was weakly inhibited in Wi38/SV40 cells and not affected in Vero cells by intracellular K$^+$ depletion. That various cell lines responded differently to the action of intracellular K$^+$ depletion is certainly caused by several factors. An important one may

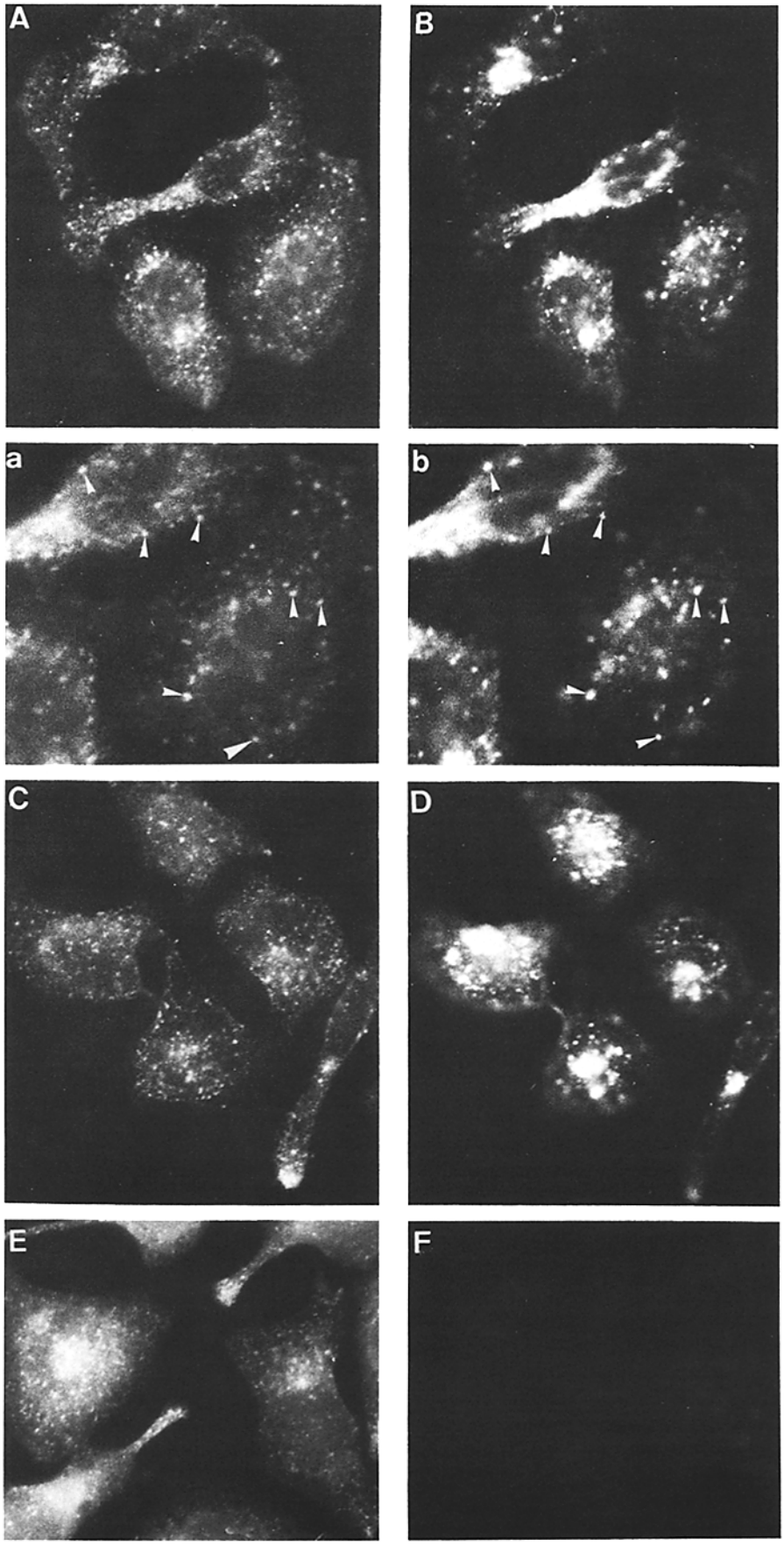

FIGURE 10 Internalization of FITC-labeled transferrin and ricin toxin in Hep_2 cells with active coated pits (*A* and *B*) or without functional coated pits (*C* and *D*). Ricin toxin was detected by indirect immunofluorescence on permeabilized cells using rabbit anti-ricin antibodies and rhodamine-labeled goat anti-rabbit IgG. Cells grown on coverslips were blocked for coated pit formation by intracellular K^+ depletion (as described in Materials and Methods. After a 5-min hypotonic shock and incubation in a K^+-free isotonic buffer (buffer B), FITC-labeled transferrin (250 nM) and ricin toxin (10^{-8} M) were added together to cells for 30 min at 37°C. The coverslips were then washed three times at 4°C with buffer B and incubated at 4°C for 15 min in the same buffer but containing 100 mM lactose to remove membrane-bound ricin toxin molecules. Cells were then processed for fixation and permeabilization (as described in Materials and Methods). Appropriate filters for visualization of FITC-labeled transferrin fluorescence (*A* and *C*) or rhodamine fluorescence (*B* and *D*) were used. Details of *A* and *B* are shown in *a* and *b*, respectively (arrows indicate the vesicles seen with both ricin toxin and transferrin). FITC-labeled transferrin or ricin toxin incubated with Hep_2 cells starved in K^+ but not permeabilized are visualized in *E* and *F*, respectively.

be the amount of polymerized clathrin present in the cell. Indeed, in preliminary experiments using a new assay to quantify the intracellular pool of polymerized clathrin (34), we have observed that Hep_2 cells had a much lower amount of polymerized clathrin than Vero cells. This element, in addition to the ability of Hep_2 cells to release K^+ more rapidly than Wi38/SV40 or Vero cells after hypotonic shock, can explain why they did efficiently block their coated pit formation after intracellular K^+ depletion.

The effects of intracellular K^+ depletion have been shown to be reversible by addition of K^+ to the culture medium (14). Thus, although it is not a physiological process, intracellular K^+ depletion, which blocks coated pit activity in certain cells, is of considerable interest to perform a fine dissection of the endocytosis process.

We have studied endocytosis of transferrin, diphtheria toxin, and ricin toxin in both K^+-containing and K^+-depleted Hep_2 cells. It is clear from our data that the cytotoxicity of diphtheria toxin depends totally on the presence of coated pits at the cell surface. Our results thus agree with a previous finding of Keen et al. (17) which showed that on cells sensitive to or resistant to diphtheria toxin, this protein clustered with α2-macroglobulin, a ligand well known to be internalized by coated pits (although the existence of specific receptors for diphtheria toxin on toxin-resistant cells is still debated [35, 36]). Keen et al. (17) did not demonstrate, however, that the toxin clustered with α2-macroglobulin was the very same fraction of molecules which successfully enters into the cytosol and inhibits protein synthesis.

Endosomes are rapidly acidified (6) and it has been shown that low pH triggers the insertion of the hydrophobic domain of diphtheria toxin B fragment into lipid membranes (13). This probably leads to the traversal of the catalytic A subunit of the diphtheria toxin molecule through the endosomal membrane into the cytosol.

Entry of the ricin toxin into the cell cytoplasm is a less clear process. Sandvig and Olsnes (16) have shown that lowering the external pH of cells incubated with ricin toxin results in a decreased toxicity of this molecule. It was therefore concluded that ricin toxin does not enter into the cells by an acidic compartment to exert its activity. It has also been shown that lysosomotropic amines such as ammonium chloride or methylamine, which are known to raise the endosomal and lysosomal pH, had opposite effects on the activities of ricin and diphtheria toxins. Indeed lysosomotropic amines inhibit the activity of diphtheria toxin, but increase the effect of ricin toxin on cells (16, 33). These results indicate that acidification of ricin toxin in the endosomal and/or lysosomal compartment destroys the biological activity of this molecule. In agreement with these observations, we have shown that after the arrest of coated pits, ricin toxin, in contrast to diphtheria toxin, retained its full activity. We observed that NH_4Cl sensitized Hep_2 cells to ricin toxin in K^+-containing medium, but not after intracellular K^+ depletion; this indicates that normally coated pits and coated vesicles are not the route through which ricin toxin reaches its cytosolic target. However, after neutralization of the acid pH present in different compartments, such as endosomes and lysosomes, ricin toxin can probably penetrate the cell using the coated pit pathway.

The possibility that, after intracellular K^+ depletion, a very small number of coated pits are still functional and are allowing some ricin toxin molecules to enter the cell cytosol is not likely. Indeed we have shown that no shift in the dose-response curve of toxicity of ricin toxin was observed between K^+-depleted and control Hep_2 cells.

In agreement with other studies we have found that internalization of ricin toxin is a very slow process. Only ~8% of cell-bound ricin toxin was internalized each 10 min at 37°C in Hep_2 cells with functional coated pits. In the same conditions, internalization of transferrin was very rapid (50% of the bound transferrin in 10 min). After intracellular K^+ depletion, Hep_2 cells internalized ~4% of the bound ricin toxin, representing half of the transported molecules with active coated pits. It is worth noting that after K^+ depletion, fluid phase uptake of Hep_2 cells was reduced but not blocked (data not shown).

We are inclined to believe that ricin toxin is usually taken up into cells by two routes of endocytosis (namely coated pits, vesicles, and another membrane which permits membrane internalization) from the following evidence: (*a*) Depletion of intracellular K^+ blocked half the internalization of [^{125}I]ricin toxin in Hep_2 cells. (*b*) Only Hep_2 cells with active coated pits were NH_4Cl-sensitized to ricin toxin. (*c*) As seen in the immunofluorescence studies performed with Hep_2 cells in K^+-containing medium, internalized ricin toxin was observed in endocytic vesicles together with transferrin molecules. We are thus inclined to believe that ricin toxin is usually taken up into cells by two routes of endocytosis, namely coated pits and coated vesicles and another mechanism which permits membrane internalization. (*d*) Upon potassium starvation, only the second mechanism of endocytosis is operative and would represent 50% of the toxin entry in normal conditions. Entry of ricin toxin into this pathway (not dependent on the coated vesicles) could allow cells to be intoxicated by this molecule. However, a direct transmembrane passage of ricin toxin cannot be ruled out. It has been already suggested by several groups that the entry of certain proteins into a cell compartment could be achieved by a mechanism different from that of coated pits and coated vesicles (37, 38).

Using neuroblastoma cells, Gonatas et al. (39) have studied the internalization of a conjugate made out of ricin toxin and horseradish peroxidase. This molecule underwent endocytosis in vesicles and cisternae in the vicinity of the Golgi apparatus. Our morphological studies indicated that in K^+-containing medium, ricin toxin was accumulated around the cell nucleus and predominantly at one pole of it. This localization is therefore in good agreement with the work of Gonatas et al. (39). After the arrest of coated pits by lowering the intracellular K^+, ricin toxin was still taken up by Hep_2 cells but was seen mainly as dense patches inside the cytosol, closely associated with the cell nucleus. We do not know if intracellular K^+ depletion affects the normal routing of ricin toxin–containing vesicles or their fusion with cytoplasmic compartments. It is clear however that the change in the morphological localization of ricin toxin seen between K^+-containing and K^+-depleted Hep_2 cells did not affect its activity.

Recently, a vesicular membrane traffic has been described for internalization of phospholipids (40) and gangliosides (41). In the case of phosphatidylcholine, Sleight and Pagano (40) have shown that after insertion of the phospholipid into the outer leaflet of the plasma membrane, the molecule was internalized within the lipid boundaries of vesicles and apparently transported to the Golgi apparatus. On the other hand, a lectin which binds glycoproteins of plasma membranes was not co-transported with phosphatidylcholine (40). Interest-

ingly, Sleight and Pagano, in the same study, have shown that ammonium chloride had no effect on the degradation of internalized phosphatidylcholine (40), which suggests that this pathway did not communicate with lysosomes.

Both glycoproteins and glycolipids are receptors for ricin toxin (42). One population of ricin toxin molecules associated with glycolipids could therefore follow the pathway described for phospholipids or glycolipids which ricin toxin linked to glycoproteins would be internalized by coated pits and normally destroyed in the lysosomal compartment. Receptor-mediated endocytosis of macromolecules could thus be accomplished by the following two (independent) mechanisms: (*a*) coated pits and coated vesicles, a fast, high-capacity system which uses transmembrane proteins as receptors (diphtheria toxin, LDL, and transferrin are typical ligands transported by this mechanism); and (*b*) another form of endocytosis which internalizes membrane-bound proteins such as ricin toxin. The nature of the receptors involved in this latter system is unknown but glycolipids could certainly be good candidates to play this role.

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REFERENCES

1. Brown, M. S., R. G. W. Anderson, and J. L. Goldstein. 1983. Recycling receptors: the round-trip itinerary of a migrant membrane protein. *Cell.* 32:663–667.
2. Helenius, A., J. Kartenbeck, K. Simons, and E. Fries. 1980. On the entry of Semliki Forest Virus into BHK 21 cells. *J. Cell. Biol.* 84:404–420.
3. Pappenheimer, A. M., Jr. 1982. Diphtheria: studies on the biology of an infectious disease. *In* The Harvey lectures. Academic Press, Inc. New York. 76:45–73.
4. Goldstein, J. L., R. G. W. Anderson, and M. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. *Nature (Lond.).* 279:679–685.
5. Helenius, A., I. S. Mellman, D. A. Wall, and A. L. Hubbard. 1983. Endosomes. *Trends Biochem. Sci.* 8:245–249.
6. Tycko, B., and F. R. Maxfield. 1982. Rapid acidification of endocytic vesicles containing $\alpha_2$macroglobulin. *Cell.* 28:643–651.
7. Yamashiro, D. J., S. R. Fluss, and F. R. Maxfield. 1983. Acidification of endocytic vesicles by an ATP-dependent proton pump. *J. Cell. Biol.* 929–934.
8. Harford, J., K. Bridges, G. Ashwell, and R. D. Klausner. 1983. Intracellular dissociation of receptor-bound asialoglycoproteins in cultured hepatocytes: a pH-mediated non lysosomal event. *J. Biol. Chem.* 258:3191–3197.
9. Dautry-Varsat, A., Ciechanover, A., and Lodish, H. F. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 80:2258–2262.
10. Marsh, M., E. Bolzau, and A. Helenius. 1983. Penetration of Semliki Forest Virus from acidic prelysosomal vacuoles. *Cell.* 32:931–940.
11. Boquet, P., M. Silvermann, A. M. Pappenheimer, Jr., and B. W. Vernon. 1976. Binding of Triton X-100 to diphtheria toxin, cross-reacting material 45 and their fragments. *Proc. Natl. Acad. Sci. USA.* 73:4449–4453.
12. Sandvig, K., and S. Olsnes. 1981. Rapid entry of nicked diphtheria toxin into cells at low pH. *J. Biol. Chem.* 256:9068–9076.
13. Kagan, B. L., A. Finkelstein, and M. Colombini. 1981. Diphtheria toxin fragment forms large pores in phospholipid bilayer membranes. *Proc. Natl. Acad. Sci. USA.* 78:4950–4954.
14. Larkin, J. M., M. S. Brown, J. L. Goldstein, and R. G. W. Anderson. 1983. Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. *Cell.* 33:273–285.
15. Gill, D. M. 1976. Protein toxins that act within cells. *Bull. Inst. Pasteur.* 74:65–84.
16. Sandvig, K., and S. Olsnes. 1982. Entry of the toxic proteins, abrin, modeccin, ricin, and diphtheria toxin into cells. *J. Biol. Chem.* 257:7504–7513.
17. Keen, J. H., F. R. Maxfield, M. C. Hardegree, and W. H. Habig. 1982. Receptor-mediated endocytosis of diphtheria toxin by cells in culture. *Proc. Natl. Acad. Sci. USA.* 79:2912–2916.
18. Karin, M., and B. Mintz. 1981. Receptor-mediated endocytosis of transferrin in developmentally totipotent mouse teratocarcinoma stem cells. *J. Biol. Chem.* 256:3245–3252.
19. Ciechanover, A., A. L. Schwartz, A. Dautry-Varsat, and H. F. Lodish. 1983. Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. *J. Biol. Chem.* 258:9681–9689.
20. Nicolson, G. L., M. Lacorbière, and W. Eckart. 1975. Qualitative and quantitative interactions of lectins with untreated and neuraminidase-treated normal, wild type, and temperature-sensitive polyoma-transformed fibroblasts. *Biochemistry.* 14:172–179.
21. Ternynck, T., and S. Avrameas. 1972. Polyacrylamide-protein immunoadsorbents prepared with glutaraldehyde. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 23:24–28.
22. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
23. Sandvig, K., S. Olsnes, and A. Pihl. 1976. Kinetics and binding of the toxic lectins abrin and ricin to surface receptors of human cells. *J. Biol. Chem.* 231:3977–3984.
24. Ray, B., and H. C. Wu. 1981. Internalization of ricin in Chinese hamster ovary cells. *Mol. Cell. Biol.* 1:552–559.
25. Reggio, H., P. Webster, and D. Louvard. 1983. Use of immunocytochemical techiques in studying the biogenesis of cell surfaces in polarized epithelia. *Methods Enzymol.* 98:379–395.
26. Ward, J. H., J. P. Kushner, and J. Kaplan. 1982. Regulation of Hela cell transferrin receptors. *J. Biol. Chem.* 257:10317–10323.
27. Ledbetter, M. L. S., and M. Lubin. 1977. Control of protein synthesis in human fibroblasts by intracellular potassium. *Exp. Cell Res.* 105:223–236.
28. Louvard, D., C. Morris, G. Warren, K. Stanley, F. Winkler, and H. Reggio. 1983. A monoclonal antibody to the heavy chain of clathrin. *EMBO (Eur. Mol. Biochem. Soc.) J.* 2:1655–1664.
29. Nicolson, G. L., and J. Blaustein. 1972. The interaction of Ricinus agglutinin with normal and tumor cell surfaces. *Biochim. Biophys. Acta.* 266:543–547.
30. Kim, K., and N. B. Groman. 1965. *In vitro* inhibition of diphtheria toxin action by ammonium salts and amines. *J. Bacteriol.* 90:1552–1556.
31. Yamaizumi, M., E. Mekada, T. Uchida, and Y. Okada. 1978. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell.* 15:245–250.
32. Sandvig, K., S. Olsnes, and A. Pihl. 1978. Binding, uptake and degradation of the toxic proteins abrin and ricin by toxin-resistant cell variants. *Eur. J. Biochem.* 82:13–23.
33. Mekada, E., T. Uchida, and Y. Okada. 1981. Methylamine stimulates the action of ricin toxin but inhibits that of diphtheria toxin. *J. Biol. Chem.* 256:1225–1228.
34. Goud, B., C. Huet, and D. Louvard. 1984. Assembled and unassembled pools of clathrin: a quantitative study of using an enzyme immunoassay. *J. Cell. Biol.* 100:521–527.
35. Hayakawa, S., T. Uchida, E. Mekada, M. R. Moynihan, and Y. Okada. 1983. Monoclonal antibodies against diphtheria toxin: effects on toxin binding and entry into cells. *J. Biol. Chem.* 258:4311–4317.
36. Didsbury, J. R., J. M. Moehring, and T. J. Moehring. 1983. Binding and uptake of diphtheria toxin by toxin-resistant Chinese hamster ovary and mouse cells. *Mol. Cell. Biol.* 3:1283–1294.
37. Montesano, R., J. Roth, A. Robert, and L. Orci. 1982. Non-coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxins. *Nature (Lond.).* 296:651–653.
38. Huet, C., J. F. Ash, and S. J. Singer. 1980. The antibody-induced clustering and endocytosis of HLA antigens on cultured human fibroblasts. *Cell.* 21:429–438.
39. Gonatas, J., A. Stiber, S. Olsnes, and N. K. Gonatas. 1980. Pathways involved in fluid phase and adsorptive endocytosis in neuroblastoma. *J. Cell. Biol.* 87:579–588.
40. Sleight, R. G., and R. E. Pagano. 1984. Transport of fluorescent phosphatidylcholine analog from the plasma membrane to the Golgi apparatus. *J. Cell Biol.* 99:742–751.
41. Spiegel, S., J. Schlessinger, and P. H. Fishman. 1984. Incorporation of fluorescent gangliosides in human fibroblasts: mobility, fate, and interaction with fibronectin. *J. Cell. Biol.* 99:699–704.
42. Olsnes, S., and A. Pihl. 1982. Toxic lectins and related proteins. *In* Molecular action of toxins and viruses. P. Cohen and S. Van Heyningen, editors. Elsevier Biomedical Press, Amsterdam. 51–105.