Acidification of the Cytosol Inhibits Endocytosis from Coated Pits

Kirsten Sandvig,* Sjur Olsnes,* Ole W. Petersen,‡ and Bo van Deurs‡

*Institute for Cancer Research at the Norwegian Radium Hospital and the Norwegian Cancer Society, Oslo, Norway; and
‡Department of Anatomy, The Panum Institute, University of Copenhagen, Copenhagen, Denmark

Abstract. Acidification of the cytosol of a number of different cell lines strongly reduced the endocytic uptake of transferrin and epidermal growth factor. The number of transferrin binding sites at the cell surface was increased in acidified cells. Electron microscopic studies showed that the number of coated pits at the cell surface was not reduced in cells with acidified cytosol. Experiments with transferrin–horseradish peroxidase conjugates and a monoclonal anti-transferrin receptor antibody demonstrated that transferrin receptors were present in ~75% of the coated pits both in control cells and in cells with acidified cytosol. The data therefore indicate that the reason for the reduced endocytic uptake of transferrin at internal pH <6.5 is an inhibition of the pinching off of coated vesicles. In contrast, acidification of the cytosol had only little effect on the uptake of ricin and the fluid phase marker lucifer yellow. Ricin endocytosed by cells with acidified cytosol exhibited full toxic effect on the cells. Although the pathway of this uptake in acidified cells remains uncertain, some coated pits may still be involved. However, the data are also consistent with the possibility that an alternative endocytic pathway involving smooth (uncoated) pits exists.

A number of different transport proteins, hormones, growth factors, toxins, and viruses enter cells by receptor-mediated endocytosis. Coated pits and coated vesicles are generally assumed to be involved in this process (for reviews, see references 33, 38, and 59). Upon entry, the ligands are transferred to endosomes where the low pH is essential for dissociation of ligands from their receptors, for release of Fe^{3+} from transferrin, and for entry into the cytosol of viruses and certain toxins.

In studies of endocytosis and its role in the mechanism of action of many ligands, it is a problem that no specific inhibitor of endocytosis is available. Although treatments with metabolic inhibitors and low temperature inhibit endocytosis, these conditions inhibit most other physiological processes in the cells as well. Recently, removal of cytoplasmic potassium was found to inhibit endocytosis of low density lipoprotein, epidermal growth factor (EGF),[1] and transferrin (29, 31, 37). However, such treatment does not efficiently inhibit the formation of coated vesicles in all cell types (37, 44). Furthermore, K^+ depletion induces cell shrinkage and inhibits anion transport (32). Alternative methods to inhibit endocytosis from coated pits are therefore warranted.

In the course of our studies of the mechanism of action of diphtheria toxin, ricin, and related toxic proteins with intracellular sites of action, we noted that acidification of the cytosol inhibited endocytic uptake of different ligands to different extents (46). Here we report that acidification of the cytosol efficiently blocks endocytosis of transferrin and EGF, whereas the uptake of the toxic plant protein ricin and of the membrane impermeant fluorescent dye, lucifer yellow, is only slightly reduced.

1. *Abbreviations used in this paper*: DMO, [^{14}-C]dimethyloxazolidine 2, 4-dione; EGF, epidermal growth factor; HRP, horseradish peroxidase; Ri-Au, ricin-gold; Tf-Au, transferrin-gold; Tf-HRP, monovalent transferrin-HRP.

Materials and Methods

Materials

[^{14}C]Dimethyloxazolidine 2, 4-dione (DMO) was obtained from New England Nuclear, Boston, MA, [^{3}H]leucine, [^{14}C]sucrose, [^{3}H]H_2O, and Na ^{125}I were from the Radiochemical Centre, Amersham, UK. Transferrin, horseradish peroxidase (HRP), pronase, EGF, 2-(*N*-morpholino)ethane sulfonic acid (MES), SPDP (3-[2 pyridyldithio]-propionic acid *N*-hydroxysuccinimide ester), Hepes, Tris, nigericin, and valinomycin were obtained from Sigma Chemical Co., St. Louis, MO. Amiloride was a gift from Merck, Sharp & Dohme, Drammen, Norway. W7 and W5 were purchased from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Transferrin was saturated with iron as described (9), and ^{125}I-labeled ligands were prepared by the iodogen method (15).

Preparation of Conjugates for Electron Microscopy

Conjugates of ricin and transferrin with colloidal gold (particle size 5–10 nm, or 20 nm) were prepared by the method of Slot and Geuze (50), and the amount of protein necessary to stabilize the colloidal gold solution was determined by the method of Horisberger and Rosset (26).

Conjugates of ricin and transferrin with HRP were prepared by the SPDP method as previously described (56, 57). After the eluted fractions were filtered through a Sephacryl-200 column, they were analyzed by SDS-PAGE, and the monovalent conjugates (i.e., those containing one transferrin or ricin molecule and one HRP molecule) were selected for further experiments.

Cells. Hep 2-cells (human laryngeal carcinoma cells with HeLa markers) were obtained from Dr. P. Boquet, Institut Pasteur, Paris, France. A431 cells were obtained from the American Tissue Type Collection, Rockville, MD.

HeLa S3, Vero, and MCF-7 cells are strains that have been growing in this laboratory for years. All cell lines used were maintained as monolayer cultures in MEM complemented with penicillin, streptomycin, and 10% (vol/vol) FCS in an atmosphere containing 5% CO_2. The day before use the cells were seeded out into 24-well disposable trays, petri dishes, or into T-25 flasks as indicated in the figure legends.

Measurement of Receptor-mediated Endocytosis of [^{125}I]Transferrin, [^{125}I]EGF, and [^{125}I]Ricin

The amounts of surface bound and internalized transferrin were measured as described by Ciechanover et al. (6). Briefly, cells were incubated with [^{125}I]transferrin for the indicated periods of time at 37°C, washed three times with ice-cold PBS, and then treated for 1 h at 0°C with 0.3 ml serum-free medium containing 0.3% (wt/vol) pronase. Then the cells and the medium were transferred to Eppendorf tubes and centrifuged for 2 min, and the radioactivity in the pellet and supernatant was measured.

Endocytosis of [^{125}I]EGF was measured as described by Haigler et al. (22). For this purpose, cells incubated with [^{125}I]EGF were washed five times with ice-cold PBS, and then incubated for 6 min in a solution containing 0.5 M NaCl, 0.2 M acetic acid, pH 2.5, to release surface bound EGF. The cells were then washed once in the same buffer and dissolved. The cell-associated radioactivity was then measured.

Endocytosis of ^{125}I-labeled ricin was measured as the amount of toxin that could not be removed from the cells with lactose, as previously described (42).

To measure the efficacy of the treatments to remove surface-bound ligands, cells on ice were treated with the ^{125}I-labeled ligands, then washed and treated as above. For transferrin and ricin <5% of the bound radioactivity remained associated with the cells after the treatment, whereas for EGF <2% radioactivity remained on the cells (data not shown).

Endocytosis of Lucifer Yellow

Endocytosis of lucifer yellow was measured essentially as described by Swanson et al. (52). Cells were incubated for 15 min with 1 mg/ml lucifer yellow, washed eight times with ice-cold PBS containing 0.1 mg/ml BSA, and then dissolved in 0.05% Triton X-100 (vol/vol) containing 0.1 mg/ml BSA. Fluorescence was measured in a fluorescence spectrometer (model LS 5; Perkin-Elmer Corp., Norwalk, CT) with excitation at 430 nm (bandwidth, 10 nm) and emission at 540 nm (bandwidth, 10 nm). Cellular uptake of lucifer yellow was strongly reduced in ATP-depleted cells.

Measurement of Cytosolic pH

The cytosolic pH was determined from the distribution of the weak acid [^{14}C]DMO as described by Deutsch et al. (12). Cells were incubated with [^{14}C]DMO (5 μCi/ml) for 5 min at 37°C, then washed five times with ice-cold PBS. The cell-associated radioactivity was then measured. The volume of extracellular liquid associated with the cells after they were washed five times was measured after incubation of the cells with [^{14}C]sucrose (5 μCi/ml). The extracellular space corresponded to 7% of the cell volume. The total volume of the cells in a well was calculated from the amount of [^{3}H]H_2O associated with cells after incubation with 1 μCi/ml [^{3}H]H_2O for 18 h.

Measurement of Cellular ATP

Cells growing in 24-well disposable trays were treated as indicated in Table II, and 250 μl 2% (wt/vol) ice-cold perchloric acid was added to each well. After 30 min the perchloric acid extract was collected and centrifuged in an Eppendorf centrifuge for 10 min. From the supernatant 200 μl was removed and mixed with 800 μl of 0.1 M Tris-EDTA, pH 7.7. Dilutions from this solution were made in the same buffer and aliquots were taken and added to a luciferin-luciferase mixture according to the procedure described by LKB (Bromma, Sweden). Emitted light was measured in an LKB Wallac luminometer 1250. A standard curve obtained by measuring samples with known amounts of ATP was used to estimate the ATP content in the sample.

Experiments with Gold and HRP Conjugates

Vero and Hep-2 cells, grown in monolayers in T-25 flasks, were washed with Hepes buffer or with the buffer used in the respective experiment and incubated with ricin-gold (Ri-Au), transferrin-gold (Tf-Au), or 10 μg/ml monovalent transferrin-HRP (Tf-HRP) under the conditions detailed in the figure legends.

In some experiments with ricin conjugates, preincubation was performed at 0–4°C with excess unlabeled ricin (0.1 mg/ml) or with 0.1 M lactose to test the specificity of conjugate binding. Similarly, in some experiments with the transferrin conjugates, preincubation at 0–4°C was performed with excess unlabeled transferrin (50 μg/ml). After the incubation with the conjugates, the cells were washed with buffer and further processed for electron microscopy as described below.

Ultrastructural Immunocytochemical Detection of Transferrin Receptors

Hep-2 cells in monolayer cultures, with and without NH_4Cl prepulsing, were incubated in 0.14 M KCl, 20 mM Hepes, pH 7, 1 mM $CaCl_2$, and 1 mM amiloride as described in the figure legends. Then the cells were washed with the same buffer at 4°C and treated with the same buffer containing a mouse monoclonal anti-human transferrin receptor antibody (diluted 1:50) (DAKO-Tf-R M734; Dakopatts, Copenhagen, Denmark) for 60 min at 4°C. After the cells were carefully washed with KCl-amiloride buffer at 4°C, they were treated with peroxidase-conjugated goat anti-mouse IgG (code No. 6450; Medac, Hamburg, FRG) in the same buffer for 60 min at 4°C. Finally, the cells were washed carefully at 4°C with the same buffer and prepared for electron microscopy. In control experiments the primary antibody was omitted from the incubation protocol.

Processing for Electron Microscopy

Cells in monolayer, treated as described above, were fixed with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, for 60 min at room temperature. In experiments with HRP conjugates and peroxidase immunocytochemistry, the cells were then washed carefully with PBS and incubated with diaminobenzidine-H_2O_2 as previously described (56, 57). The cells were then scraped off the flasks and centrifuged in buffer. Pellets were postfixed with OsO_4, treated with 1% uranyl acetate in distilled water, embedded in Epon, cut at ~50 nm, and examined in a JEOL 100 CX electron microscope as previously described (56, 57). The frequency of coated pits at the cell surface was determined as reported elsewhere (31).

Results

To test if endocytosis of ligands that enter cells from coated pits is influenced by the pH in the cytosol, we studied the internalization of transferrin and EGF.

Both of these ligands have been shown to enter cells by endocytosis from coated pits (13, 14, 20, 21, 34). It should be noted, however, that a recent report suggests that EGF is also taken up from uncoated pits (24).

EGF stimulates internalization of the hormone–receptor complex which is then degraded (8), while the receptors for transferrin seem to be internalized constitutively, i.e., internalization occurs whether or not ligand is bound, and the receptor is then recycled to the cell surface (3).

We have used three different methods to acidify the cytosol. In the first method, which is best tolerated by the cells, we preincubate the cells with NH_4Cl to load them with NH_4^+, and then transfer the cells to medium without NH_4Cl (4). Ammonium ions are in equilibrium with NH_3, which is membrane permeant. Therefore, upon removal of extracellular NH_4Cl, NH_3 will rapidly diffuse out of the cells, while the protons are left behind. After such treatment the Na^+/H^+ exchanger is normally activated, and the cytosolic pH is rapidly regulated back to neutrality (36, 46). However, the Na^+/H^+ exchanger can be inhibited by amiloride or blocked if the cells are transferred to Na^+-free buffer. Under such conditions, low intracellular pH can be maintained for a considerable period of time.

We have also acidified the cytosol by a method based on the ability of certain weak acids, such as acetic acid, to rapidly penetrate the cell membrane in its undissociated form.

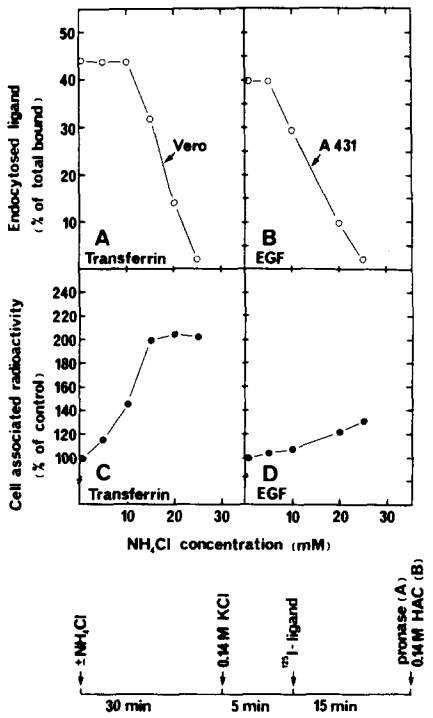

Figure 1. Effect of NH_4Cl prepulsing on binding and endocytosis of transferrin and EGF. Vero cells growing in 24-well disposable trays (*A*, *C*) and A431 cells growing in petri dishes (35-mm diam) (*B*, *D*) were incubated for 30 min at 37°C in Hepes medium (pH 7.0) with the indicated concentrations of NH_4Cl. The medium was then removed, and 0.2 ml (*A*, *C*) or 0.5 ml (*B*, *D*) 0.14 M KCl, containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM amiloride, and 20 mM Hepes (pH 7.0) were added. After 5 min further incubation ^{125}I-labeled transferrin (200 ng/ml, 38,600 cpm/ng) (*A*, *C*) and EGF (7 ng/ml, 15,000 cpm/ng) (*B*, *D*) were added, and cell-bound (*C*, *D*) and endocytosed (*A*, *B*) transferrin and EGF were measured after 10 or 15 min respectively, as described in Materials and Methods.

Once in the cytosol, the acid dissociates, thereby lowering the cytosolic pH (2, 40, 41).

A third way to manipulate the cytosolic pH is based on the ability of nigericin to carry out electroneutral K^+/H^+ exchange. Therefore, when the potassium concentration is equal on the two sides of the plasma membrane, the intracellular pH is clamped at the same value as that in the external buffer (53). This method allows acurate adjustments of the internal pH.

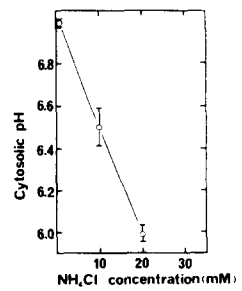

Figure 2. Effect of NH_4Cl prepulsing on cytosolic pH. Vero cells growing in 24-well disposable trays were incubated for 30 min at 37°C in Hepes medium (pH 7.0) with and without NH_4Cl as indicated. Then the medium was removed and 0.14 M KCl, containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM amiloride, and 20 mM Hepes (pH 7.0) was added. After a 5-min incubation at 37°C [^{14}C]DMO was added, the distribution of DMO was measured after 5 min further incubation, and the cytosolic pH was calculated as described in Materials and Methods.

Effect of NH_4Cl Prepulsing on Endocytosis of Transferrin, EGF, Ricin, and Lucifer Yellow

In a first set of experiments we lowered the pH in the cytosol by NH_4Cl preloading and subsequent removal, and measured the effect on the endocytosis of ^{125}I-labeled transferrin and EGF. As shown in Fig. 1, *A* and *B*, the internalization of both ligands was strongly reduced after preincubation with 25 mM NH_4Cl, whereas preincubation with 10 mM NH_4Cl had little or no effect. The reduction of endocytosis was not due to the incubation with NH_4Cl as such. Thus, there was no effect on the uptake of EGF and transferrin when NH_4Cl was present throughout the experiment (data not shown).

In experiments with the fluorescent probe, BCECF (2′, 7′-bis-[2-carboxyethyl]-5[and -6]carboxyfluorescein, acetoxymethyl ester), we have earlier found that when Vero cells are preincubated at pH 7.0 with 25 mM NH_4Cl and then transferred to medium without NH_4Cl, the pH in the cytosol is reduced at least to pH 6.0, which represents the limit of detection with this method (45). To measure the internal pH under the conditions here used, we measured the distribution of the weak acid [^{14}C] DMO (12) after incubation of cells with NH_4Cl and subsequent transfer to buffer without NH_4Cl. The data presented in Fig. 2 show that the internal pH decreased linearly with the concentration of NH_4Cl present during the preincubation, indicating that the internal buffering capacity is essentially constant between pH 6 and 7. This has also been found in other systems (4). By comparing the data in Figs. 1 and 2 it appears that to reduce the endocytosis, the internal pH must be reduced to values below pH 6.5.

It should be noted that the concentration of NH_4Cl required during the preincubation varied somewhat (25–40 mM) from experiment to experiment. The reason for this is not known. It is therefore necessary in each experiment involving electron microscopy (see below) to measure the endocytic uptake of transferrin or EGF in a parallel experiment.

Although acidification of the cytosol inhibited the endocytic uptake of transferrin, it increased the total amount of transferrin associated with the cells (Fig. 1 *C*). Scatchard analysis of the binding data indicated that the increased binding is due to an increased number of transferrin receptors on the cell surface (Fig. 3). Thus, acidification of the cytosol after prepulsing with NH_4Cl increased the number of transferrin molecules bound per Vero cell from 100,000 to 175,000. Maximal increase in binding was obtained 5–10 min after the transfer to NH_4Cl-free buffer (data not shown). The increase in the number of transferrin receptors was dependent on Ca^{2+} in the medium and could be inhibited by the calmodulin antagonists trifluoperazine and W7. W5, an inactive analogue of W7, had no effect (data not shown). The effect of acidification of the cytosol on the amount of EGF associated with the cells was much less, but also in this case a slight increase was observed (Fig. 1 *D*).

The strong reduction in endocytosis occurring after incubation with 25–40 mM NH_4Cl was rapidly reversible when the internal pH was normalized. Thus, when cells that had been acidified by an NH_4Cl prepulse were incubated with transferrin in the presence of Na^+ to allow pH regulation by Na^+/H^+ exchange to occur, no reduction in endocytic uptake of transferrin was observed. In fact, the total amount of endocytosed transferrin was in that case higher than in con-

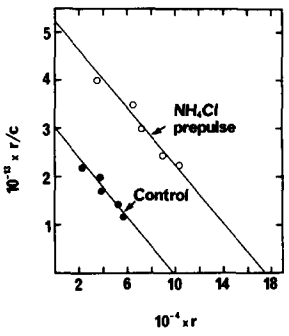

Figure 3. Equilibrium binding of ^{125}I-labeled transferrin to Vero cells. Vero cells growing in 24-well disposable trays were incubated for 30 min at 37°C in Hepes medium (pH 7.0) with and without 25 mM NH_4Cl. The medium was then removed and 0.14 M KCl, containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM amiloride, and 20 mM Hepes (pH 7.0) was added. After a 5-min incubation at 37°C, the cells were chilled to 0°C, increasing concentrations of ^{125}I-labeled transferrin (6,300 cpm/ng) were added in the absence and presence of 100 μg/ml unlabeled transferrin, and the cells were incubated for 2 h at 0°C. They were then washed three times in cold PBS, dissolved in 0.1 M KOH, and the radioactivity associated with the cells was measured. The amount of transferrin bound per cell was calculated, the amount of transferrin bound in the presence of excess unlabeled transferrin was subtracted, and the data were plotted according to Scatchard (47). r, number of transferrin molecules bound per cell; c, molar concentration of free transferrin.

trol cells (data not shown). This is probably due to the increase in surface binding sites that had taken place while the cytosol was acidified.

We also studied the effect of NH_4Cl prepulsing on the endocytic uptake of ^{125}I-labeled ricin in Vero cells, Hep 2 cells, MCF7 cells, and A431 cells. In contrast to the results obtained with transferrin and EGF, endocytosis of ricin was only slightly reduced when the cytosol was acidified (Table I). This was confirmed by electron microscopic studies shown below. Also, there was no large effect on the endocytosis of the fluorescent fluid phase marker, lucifer yellow, when the cytosol was acidified by prepulsing of the cells with NH_4Cl (Table I). In contrast, the uptake of lucifer yellow was strongly inhibited in ATP-depleted cells or when the temperature was reduced to 4°C (data not shown).

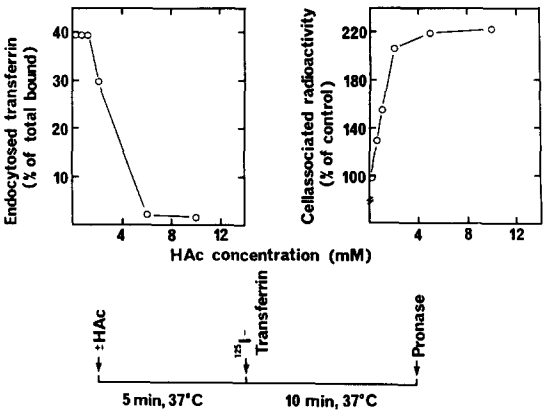

Figure 4. Ability of acetic acid to decrease endocytosis of transferrin and to increase the total amount of bound transferrin. Vero cells growing in 24-well disposable trays were incubated for 5 min at 37°C in Hepes medium (0.2 ml, pH 5.0) with and without the indicated concentrations of acetic acid. ^{125}I-Transferrin (200 ng/ml, 38,600 cpm/ng) was then added and, after a 10-min incubation, surface bound and endocytosed transferrin was measured as described in Materials and Methods.

Table I. Effect of Acidification of the Cytosol on the Ability of Vero, Hep 2, MCF 7, and A431 Cells to Endocytose [^{125}I]Ricin and Lucifer Yellow

Treatment*	Endocytic uptake of [^{125}I]ricin‡	Endocytic uptake of lucifer yellow§
	% of control	*% of control*
Prepulsing with 25 mM NH_4Cl (Vero)	82 ± 15	84 ± 12
Prepulsing with 25 mM NH_4Cl (Hep 2)	69 ± 17	
Prepulsing with 25 mM NH_4Cl (MCF 7)	65 ± 10	
Prepulsing with 25 mM NH_4Cl (A431)	79 ± 10	
5 mM acetic acid in Hepes medium, pH 5.0 (Vero)	78 ± 8	
0.14 M KCl, nigericin, valinomycin, pH 6.0 (Vero)	30 ± 14	25 ± 9

* Cells growing in monolayer cultures were treated as described in the legends to Figs. 1, 4, and 5.
‡ The cells were incubated with [^{125}I]ricin (50 ng/ml, 25,000 cpm/ng) for 15 min at 37°C. Endocytosed ricin was then determined as described in Materials and Methods. The data indicate average ±SD.
§ The uptake of lucifer yellow was measured fluorometrically as described in Materials and Methods.

Ability of Acetic Acid to Inhibit Endocytosis of Transferrin

In experiments where we acidified the cytosol of Vero cells by incubation with acetic acid, we obtained essentially the same results as when the acidification was carried out by NH_4Cl prepulsing. Thus, the endocytosis of transferrin was strongly reduced (Fig. 4 *A*), and the total amount of transferrin associated with cells was increased (Fig. 4 *B*). The extent of the increase in binding varied between different experiments. Furthermore, there was only a slight reduction in uptake of ^{125}I-labeled ricin (Table I).

Regulation of Cytosolic pH by Incubation of Cells with Nigericin in Isotonic KCl

When cells are incubated in isotonic KCl containing nigericin, the pH in he cytosol will be the same as that in the surrounding medium (53). Valinomycin was added to ensure a high K^+ permeability. It is shown in Fig. 5, that also when the pH in the cytosol was lowered by this method, the endocytosis of transferrin and EGF was inhibited in all of the four cell lines tested. Consistent with the results described above, the pH must be reduced below pH 6.5 to inhibit the endocytosis efficiently.

It should be noted that after incubation with isotonic KCl in the presence of valinomycin and nigericin and then transfer to normal medium, the endocytosis was not restored as fast as when the cytosol had been acidified by the two other methods. This could be due to incomplete removal of the ionophores. Furthermore, this method also reduced the endocytic uptake of cell-bound ricin and of lucifer yellow (Table I) although not to the same extent as the uptake of transferrin and EGF (Fig. 5). Apparently, the method demonstrated in Fig. 5 has a general toxic effect on the cells (see below). Altogether it may be concluded that acidification of the cytosol by three different methods had essentially the same effect on the uptake of the markers here studied.

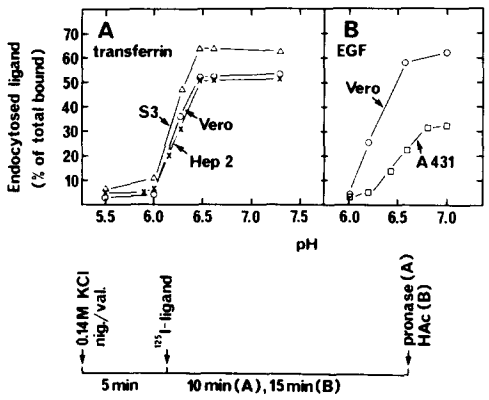

Figure 5. Effect of cytosolic pH on endocytosis of transferrin and EGF. The cell lines used were growing in 24-well disposable trays for measurements of transferrin endocytosis (*A*) and in petri dishes (35 mM) for studies of EGF endocytosis (*B*). The growth medium was removed and 0.2 ml (*A*) or 0.5 ml (*B*) 0.14 M KCl, containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 μM nigericin, 10 μM valinomycin, and adjusted to the indicated pH values with Tris, were added. After a 5-min incubation at 37°C, ^{125}I-labeled transferrin (200 ng/ml, 38,600 cpm/ng and EGF (7 ng/ml, 15,000 cpm/ng) were added, and the amount of endocytosed ligand was measured 10 min (transferrin) or 15 min (EGF) later, as described in Materials and Methods.

Effect of Acidification of the Cytosol on the Metabolic Activity of the Cells

To test if acidification of the cytosol has a general toxic effect on the cells, we transferred the cells back to normal medium to allow normalization of the internal pH to occur. In a first set of experiments, we added [^{3}H]leucine to the cells 15 min later and measured their ability to incorporate the labeled amino acid during 10 min. The data in Table II show that in cells that had been treated with nigericin, valinomycin, and isotonic KCl, the rate of protein synthesis was strongly reduced, whereas in cells that had been acidified by the two other methods, the rate of protein synthesis was only reduced to approximately half the control value.

When we measured the rate of [^{3}H]leucine incorporation 12 h later, the rate was close to that in the control cells. Clearly, acidification of the cytosol had only a transient effect on protein synthesis and did not affect cell survival, with the possible exception of acidification by treatment with nigericin, valinomycin, and KCl.

When the ATP level of cells is reduced to <10% of the control level, endocytosis in general is blocked (51) although exceptions may exist (19, 30). As shown in Table III, the ATP-level was not strongly reduced by any of the three methods here used to acidify the cytosol. Altogether, it may be concluded that acidification of the cytosol by NH_4Cl prepulsing or by treatment with acetic acid, does not induce any permanent damage to the cells, while the method of pH-clamping with KCl and ionophores may have a slight toxic effect.

Electron Microscopical Observations

To visualize the effect of low pH in the cytosol on endocytosis, we carried out ultrastructural studies. In the following we have taken as coated pits any structure ranging from a coated portion of the plasma membrane to a coated vesicular profile located less than three profile diameters away from the plasma membrane (see also reference 31). Both in Vero and Hep-2 cells coated pits were present at the cell surface at approximately the same frequency in control incubations and in experiments where the cytosol was acidified (see Figs. 6 and 7, and Table IV). Also smooth pits or invaginations were observed under both control and experimental conditions. Their size varied considerably, from ~50–70 nm (being the most frequent diameter) and up to ~250 nm (Fig. 6). Serial section analysis revealed that many endosome-like vacuolar structures in the peripheral cytoplasm were in fact surface connected, thus representing pits (Fig. 6). The frequency of the various smooth pits was, however, difficult to determine. In the first place, the number (particularly that of the small ones) varied considerably from region to region of the cells. Furthermore, it is meaningless to discriminate shallow pits from simple invaginations due to irregular cell surface geometry. However, the general impression obtained was that there are from one to eight times as many smooth pits (all sizes included) than coated pits (see also reference 31). The acidified cells showed a somewhat altered morphology, most notably in the Golgi complexes, which exhibited a marked swelling and vesiculation (not shown).

We have earlier shown that when Ri-Au or Ri-HRP conjugates are added to cells at 0–4°C, ricin binding sites are

Table II. Effect of Acidification of the Cytosol on the Ability of Vero Cells to Incorporate [^{3}H]Leucine

Treatment	Incorporation of [^{3}H]leucine*	
	Time after treatment	
	15 min	12 h
	% of control	
None (Hepes medium, pH 7.0)	100	100
Prepulsing with 25 mM NH_4Cl	44	100
0.14 M KCl, nigericin, valinomycin, pH 7.0	7	73
0.14 M KCl, nigericin, valinomycin, pH 6.0	7	72
Hepes medium, pH 5.0	63	119
Hepes medium, pH 5.0, 2 mM acetic acid	55	123
Hepes medium, pH 5.0, 5 mM acetic acid	44	122

* The cells were incubated with [^{3}H]leucine for 10 min in Hepes-containing medium (pH 7.0) without unlabeled leucine and the incorporation of [^{3}H]leucine was measured as described in Materials and Methods.

Table III. Effect of Acidification of the Cytosol on the Cellular Content of ATP in Vero Cells

Treatment	Concentration of ATP*
	% of control
None (Hepes medium, pH 7.0)	100
Prepulsing with 25 mM NH_4Cl	71
0.14 M KCl, nigericin, valinomycin, pH 6.5	76
0.14 M KCl, nigericin, valinomycin, pH 6.0	73
Hepes medium, pH 5.0	84
Hepes medium, pH 5.0, 10 mM acetic acid	66

* The concentration of ATP was measured as described in Materials and Methods 15 min after acidification of the cytosol. The data are given as percent of the control value, i.e., the content of ATP in cells kept in Hepes medium, pH 7, alone.

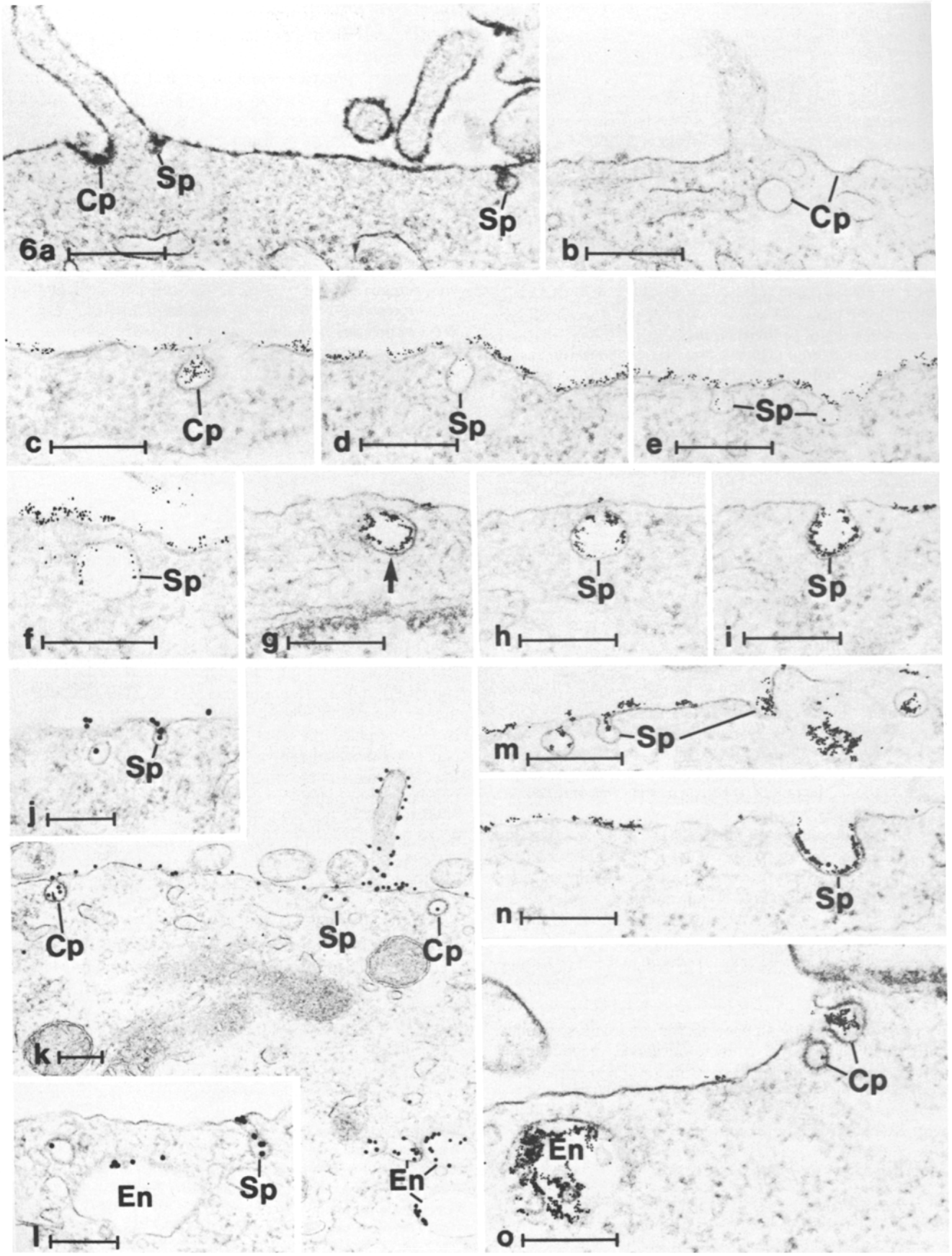

Figure 6. Electron micrographs of Vero cells incubated with various ricin conjugates. *a* shows a portion of a cell incubated with monovalent Ri-HRP conjugate at 4°C. The conjugate binds evenly to the cell surface and also labels coated pits (*Cp*) and smooth pits (*Sp*). *b* represents

evenly distributed all over the cell surface (55, 57). Control experiments showed that the binding of these markers was specific. Thus, preincubation with excess of unlabeled ricin or with 0.1 M lactose prevented the binding of these conjugates to the cells (Fig. 6 *b*, and data not shown).

The data in Fig. 6 show that both conjugates were found in practically all coated pits as well as in smooth pits of various sizes, and no differences could be established between control and acidified cells.

At 37°C the amount of surface labeling decreased due to internalization of the conjugates, but it could still be observed in coated and smooth pits (Fig. 6). The conjugates were also present in endosomal elements (Fig. 6), approximately to the same extent both in control and acidified cells. The ultrastructural observations are therefore in agreement with the biochemical data.

In our first attempts to visualize binding and uptake of transferrin, a conjugate of transferrin and gold (Tf-Au) was used. The conjugate was found at the cell surface both as single particles and as aggregates of various sizes. The conjugate showed a preferential localization to coated pits and their surroundings, while smooth pits were labeled only on occasions. This pattern was found in both control and acidified cells. However, since preincubation with unlabeled transferrin did not completely prevent surface labeling, particularly by the larger aggregates, we considered the Tf-Au conjugate as an unreliable marker of the surface distribution of transferrin receptors.

To circumvent the problem of unspecific binding we carried out further experiments with a monovalent Tf-HRP conjugate. The binding of this conjugate could be prevented by preincubation with unlabeled transferrin (data not shown). In agreement with previous reports on the transferrin receptor distribution (23, 25) the HRP reaction product was mainly found close to or within coated pits, leaving the smaller smooth pits largely unlabeled (Fig. 7). The largest smooth pits were never labeled. The frequency of Tf-HRP labeled coated pits in nonacidified and acidified Hep-2 cells was approximately the same (Table V).

At 37°C Tf-HRP was frequently observed in endosomes in control cells (Fig. 7). In contrast, almost no endosomal structures were labeled in the acidified cells. This was as expected since the parallel Hep-2 culture flasks (same series of experiments) revealed a decrease in endocytosed [^{125}I]transferrin from 60% of the total bound transferrin (10 min after addition of transferrin) in control cells to ~5% in acidified cells (not shown).

In another set of experiments we visualized transferrin receptors by using immunoperoxidase cytochemistry and a monoclonal mouse anti-human transferrin receptor antibody. Because of the specificity of the antibody in these experiments we used exclusively Hep 2 cells, which are of human origin. This approach gave approximately the same results as described above for the Tf-HRP conjugate (Fig. 7 and Table V).

Immunocytochemical reaction product clearly was most distinct within or close to coated pits (Fig. 7). Smaller smooth pits were sometimes weakly labeled, but no reaction product was observed in distinct, large pits. The labeling was almost completely prevented when the primary antibody was omitted from the incubation protocol. It should also be noted that similar experiments carried out with other human cell types (39) gave identical results (data not shown).

From the ultrastructural studies the following conclusions can be made. (*a*) Molecules binding ricin are distributed with a high frequency all over the cell surface including coated pits and smooth pits and invaginations of various sizes, whereas transferrin receptors are preferentially localized to coated pit regions. (*b*) Coated pits are present in acidified cells with approximately the same or at a slightly higher frequency than in control cells. (*c*) Also in acidified cells the coated pits contain transferrin receptors.

Toxic Effect of Ricin Endocytosed at Normal and Acidic Internal pH

Ricin is a toxic protein that exerts its action on the ribosomes (38). We have earlier shown that endocytic uptake of ricin is involved in the mechanism of entry into the cytosol (43, 45). Since the endocytic uptake of ricin is not strongly reduced by acidification of the cytosol, we decided to study if ricin endocytosed under such conditions can intoxicate cells. In these experiments we used the NH_4Cl prepulse method to acidify the cytosol. Toxin was added and endocytosis was allowed to proceed for 15 min. Then the cells were transferred to normal medium containing neutralizing amounts of antitoxin to inactivate any toxin present at the cell surface. After incubation overnight the ability of the cells to incorporate [^{3}H]leucine was measured. The data in Fig. 8 show that protein synthesis in cells treated in this way was inhibited to the same extent as in control cells, where the endocytic uptake of the toxin occurred at normal internal pH.

Discussion

The major new observation in the present study is that acidification of the cytosol to values below pH 6.5, selectively inhibits endocytosis of transferrin and EGF, while it only slightly reduces the endocytic uptake of ricin and lucifer yellow. This conclusion is based on three different methods of acidification and the endocytosis is evaluated both by biochemical and morphological methods.

To explain why acidification inhibits endocytosis at least three possibilities must be considered: (*a*) At low intracellu-

a control experiment where the Ri-HRP binding was prevented by incubation with 0.1 M lactose. *c–f* represent cells incubated with Ri-Au (5–10 nm) at 4°C. The even distribution of the frequent ricin binding sites at the cell surface is evident, and coated (*Cp*) and smooth (*Sp*) pits of various sizes are also labeled. Since incubation has been performed at 4°C, the labeled profile in *f* must communicate with the cell surface at another plane of sectioning. *g–i* show cells incubated with Ri-Au (5–10 nm) at 4°C and thereafter for 15 min at 37°C. The arrow in *g* indicates a labeled profile, which may be a peripheral endosome or a pit communicating with the cell surface at another plane of sectioning. *h* and *i* are section Nos. 2 and 4 of a series of consecutive sections showing a typical large, smooth pit (*Sp*) with Ri-Au. *j–o* show cells acidified by prepulsing with 25 mM NH_4Cl (see legends to Fig. 1) and then incubated with Ri-Au (20 nm in *j–l*, and 5–10 nm in *m–o*) for 30 min at 37°C. Coated pits (*Cp*), smooth pits of various sizes (*Sp*), and endosomes (*En*) contain the conjugate. Bars, 0.25 μm.

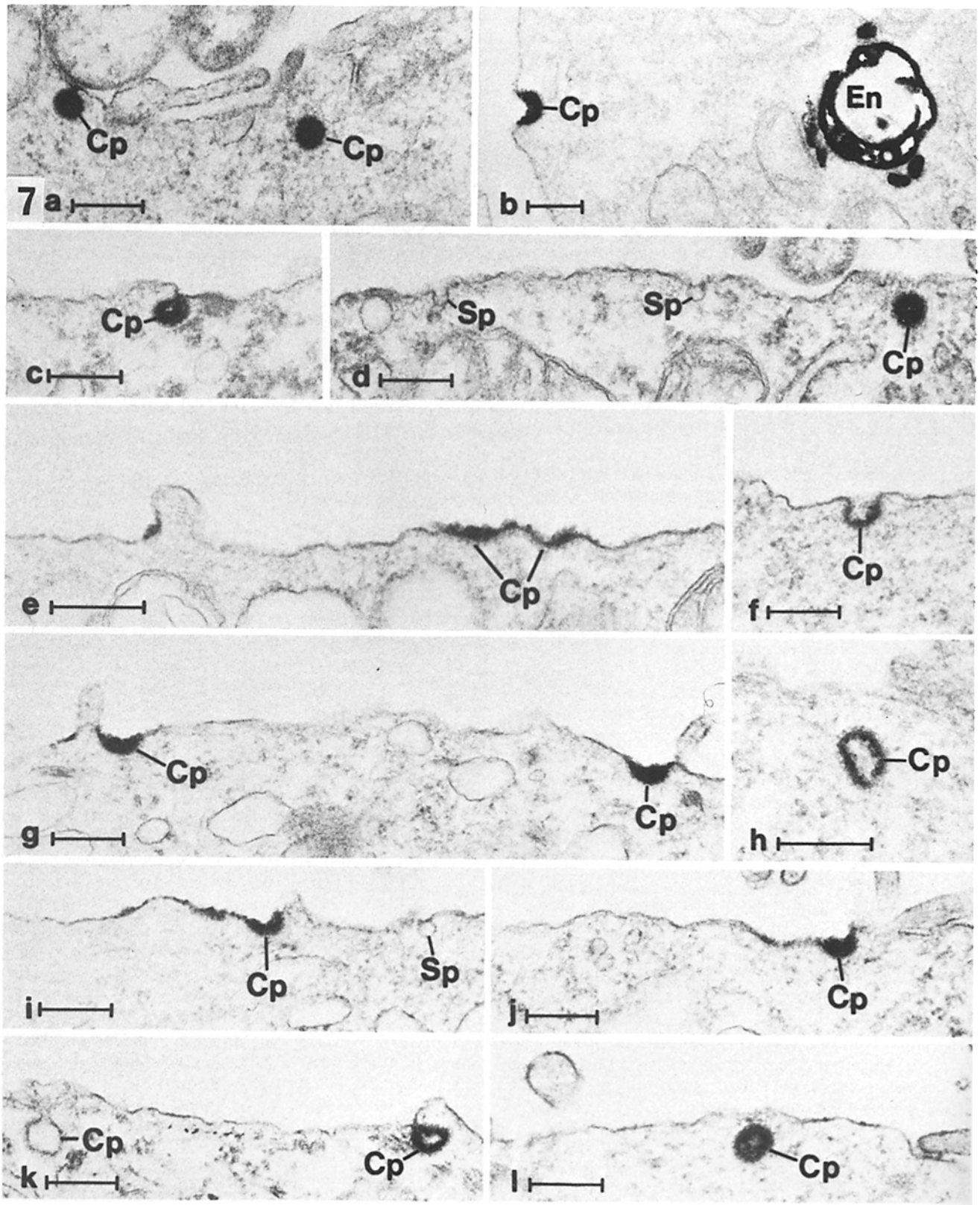

Figure 7. Electron micrographs of Vero and Hep-2 cells incubated with Tf-HRP or anti-transferrin receptor antibody. *a* shows a Vero cell incubated at 4°C with monovalent Tf-HRP. *b* represents a Hep-2 cell incubated with Tf-HRP for 10 min at 37°C. *c* and *d* are Hep-2 cells acidified by prepulsing with 40 mM NH_4Cl and then incubated with Tf-HRP for 10 min at 37°C. In all cases Tf-HRP binds preferentially to coated pits (*Cp*). Smooth pits (*Sp*) are unlabeled. In *b* is also shown a Tf-HRP containing endosome (*En*). *e–l* shows Hep-2 cells incubated at 4°C with a mouse monoclonal anti-human transferrin receptor antibody, and thereafter with peroxidase-conjugated goat anti-mouse IgG. *e–h* represent nonacidified cells, and *i–l* represent cells acidified by prepulsing with 40 nm NH_4Cl. Transferrin receptors are in both cases localized close to or within coated pits (*Cp*). Smooth pits (*Sp*) are unlabeled. Bars, 0.25 μm.

Table IV. Quantitative Data on the Number of Coated Pits at the Cell Surface

Cell type	Experiment	Coated pits	Amount of surface analyzed
		No./mm	*mm*
Vero	Control	36	2.6
	Acidified (NH_4Cl prepulsing)	58	3.1
Hep-2	Control	52	1.7
	Acidified (NH_4Cl prepulsing)	59	1.5

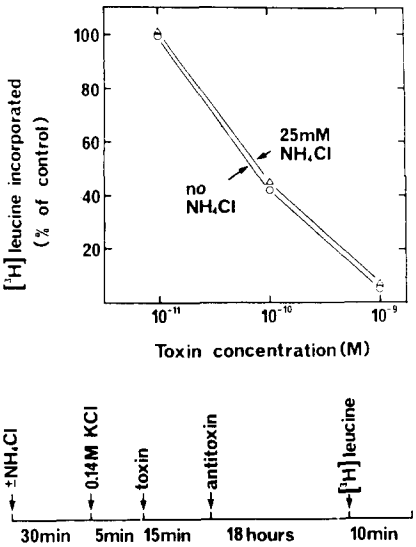

Figure 8. Toxic effect of ricin endocytosed at normal and acidic internal pH. Vero cells growing in 24-well disposable trays were incubated for 30 min at 37°C in Hepes medium (pH 7.0) with and without 25 mM NH_4Cl. The medium was then removed and 0.14 M KCl, containing 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM amiloride, and 20 mM Hepes (pH 7.0) was added. After a 5-min incubation at 37°C increasing concentrations of ricin were added and, after 15 min further incubation, growth medium containing neutralizing amounts of antiricin was added. The cells were then incubated overnight and finally the ability of the cells to incorporate [^{3}H]leucine was measured as described in Materials and Methods.

lar pH certain receptors (e.g., transferrin and EGF receptors) could be selectively prevented from gaining access to coated pits and subsequent internalization. (*b*) At low internal pH the formation of coated pits at the cell surface could be inhibited as in experiments with K^+-depletion after hypotonic shock (28–31). (*c*) Low internal pH may inhibit coated pits from pinching off from the cell surface.

The first two of these possibilities are contradicted by our ultrastructural observations. Thus, coated pits were present at the cell surface of acidified cells in approximately the same number as in control cells, and in both cases the majority of the coated pits contained transferrin receptors. Therefore, our results are in favor of the third possibility, that acidification prevents pinching off of coated pits.

It remains uncertain how ricin and lucifer yellow were internalized in acidified cells. One possibility is that these molecules were taken up by a population of newly formed coated pits and vesicles that were not immobilized at the cell surface during acidification in contrast to those containing transferrin receptors. However, our findings are also compatible with the possibility that an alternative (uncoated) endocytic pathway is responsible for the uptake of most ricin and lucifer yellow. A similar conclusion was recently reached by Moya et al. (37) and Madshus et al. (31) in studies of internalization of transferrin and ricin in K^+-depleted cells.

The crucial and still unanswered question now is: what is the structural equivalent to such an alternative endocytic pathway? Obviously, one must look for surface pits containing binding sites for ricin, but not for transferrin. We found that the smooth pits, in particular the larger ones, represent such structures. At least four recent electron microscopic studies using ferritin or gold probes have suggested that smooth surface pits are involved in endocytosis. Thus, Huet et al. (27) found that IgG-ferritin binding to the major histocompatibility surface antigens of cultured human fibroblasts was localized to smooth pits rather than coated pits and that the ligand was subsequently internalized. Furthermore, Montesano et al. (35) found that tetanus toxin and cholera toxin bound to smooth pits rather than to coated pits and were subsequently internalized by smooth vesicles in cultured liver cells. Hopkins et al. (24) found that while transferrin was taken up via coated pits and vesicles in cultured epitheloid A431 cells, EGF induced formation of smooth pits which then internalized the ligand. Furthermore, Ghitescu et al. (18) found that smooth pits and vesicles were involved in receptor-mediated transcytosis of albumin-Au in capillary endothelial cells.

Table V. Frequency of Coated Pits in Hep-2 Cells Binding Monovalent Tf-HRP and Mouse Monoclonal Anti-Human Transferrin Receptor Antibody (Anti-Tf-R)

	Nonacidified cells	Acidified cells (NH_4Cl prepulsing)
Tf-HRP	62% (n = 65)	70% (n = 33)
Anti-Tf-R	81% (n = 68)	75% (n = 32)

However, criticisms against an endocytic role of the small, smooth surface pits have been raised in studies analyzing thin serial sections of endothelial cells (5, 16). These studies showed that practically all structures believed to be endothelial vesicles were in fact surface connected and therefore probably not involved in endocytosis. Also, that a given small, smooth surface pit contains a certain ligand does not necessarily imply that it represents a forming endocytic vesicle. Previous studies have shown that recycling vesicles being in the process of exocytosis may have the same size and appearance (54). It should also be noted that the appearance of secretory vesicles (the constitutive pathway) coming from the Golgi complex is largely unknown, and some small, smooth pits at the cell surface could therefore represent secretory vesicles. Altogether, it appears that the small smooth pits at the cell surface may very well represent a heterogeneous population of structures with various, largely nonendocytic, but hitherto unknown functions.

The larger smooth pits (Fig. 6, *f–i*, and *n*) at the cell surface represent an alternative to the small, smooth pits as can-

didates for structures involved in endocytosis of ricin (see also reference 55). Although it is impossible at present to prove their role in endocytosis, it is obvious that even a relatively few such structures would engulf sufficient material to quantitatively be of great importance in endocytosis. However, further studies are still necessary to prove or disprove this concept.

Schlossman et al. (49) found that both spontaneous and ATP-dependent release of clathrin from coated vesicles were inhibited at low pH. However, as shown here, inhibition of endocytosis at low pH is not due to accumulation of coated vesicles in the cytosol. Rather low pH in the cytosol appears to prevent coated pits from pinching off and forming coated vesicles.

When the cytosol was acidified we found a higher amount of transferrin bound at the cell surface than in control cells. This increase is probably not due to inhibition of endocytosis, since increased binding was already observed at cytosolic pH values that were higher than those required to block endocytosis. Also, at 37°C the total amount of transferrin associated with cells having low cytosolic pH was higher than in control cells incubated with transferrin at 37°C. Recently, redistribution of internal transferrin receptors to the cell surface has been shown to occur upon addition to cells of serum, insulin, insulin-like growth factors, EGF, and platelet-derived growth factor (10, 11, 58). Similarly to the results described in the present paper, Ca^{2+} in the medium was required for the serum-induced redistribution. It has been suggested that calmodulin or another Ca^{2+}-dependent regulatory protein is involved in an exocytic reaction. Shecter et al. (48) found that trifluoperazine inhibits insulin-induced exocytosis of glucose transporters. Furthermore, both trifluoperazine and W7 inhibit secretion in *Paramecium* (17). Recently, Adelsberg and Al-Awqati (1) showed that internal low pH induces exocytosis of proton pumps in the turtle bladder epithelium by a Ca^{2+}-dependent mechanism, and it is possible that the increased number of transferrin receptors at the cell surface is due to a related process. Our finding that trifluoperazine and W7 prevent the acidification-induced increase in transferrin binding capacity is in agreement with this suggestion.

In conclusion, we have shown that acidification of the cytosol selectively inhibits the internalization of certain molecules by inhibiting pinching off of coated pits, whereas the uptake of other molecules was only slightly reduced. Our data are compatible with the notion that two endocytic pathways may operate in parallel, and that only one of them, the coated pit pathway, is inhibited at low intracellular pH.

We thank Kai Simons for critical reading of the manuscript. We also thank Tove Lie Berle, Jorunn Jacobsen, Keld Ottosen, and Kirsten Pedersen for technical assistance.

This work was supported by the NOVO Foundation.

Received for publication 9 March 1987, and in revised form 1 April 1987.

References

1. Adelsberg, J. van, and Q. Al-Awqati. 1986. Regulation of cell pH by Ca^{2+}-mediated exocytotic insertion of H^+-ATPases. *J. Cell Biol.* 102: 1638–1645.
2. Aubert, L., and R. Motais. 1974. Molecular features of organic anion permeability in ox red blood cell. *J. Physiol. (Lond.).* 246:159–179.
3. Bleil, J. D., and M. S. Bretscher. 1982. Transferrin receptor and its recycling in HeLa cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:351–355.
4. Boron, W. F. 1977. Intracellular pH transients in giant barnacle muscle fibers. *Am. J. Physiol.* 233:C61–C73.
5. Bundgaard, M., P. Hagman, and C. Crone. 1983. The three-dimensional organization of plasmalemmal vesicular profiles in the endothelium of rat heart capillaries. *Microvasc. Res.* 25:358–368.
6. 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.
7. Cohen, S., G. Carpenter, and L. King, Jr. 1980. Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. *J. Biol. Chem.* 255:4834–4842.
8. Das, M., and C. F. Fox. 1978. Molecular mechanism of mitogen action: processing of receptor induced by epidermal growth factor. *Proc. Natl. Acad. Sci. USA.* 75:2644–2648.
9. Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 80:2258–2262.
10. Davis, R. J., A. Corvera, and M. P. Czech. 1986. Insulin stimulates cellular iron uptake and causes the redistribution of intracellular transferrin receptors to the plasma membrane. *J. Biol. Chem.* 261:8708–8711.
11. Davis, R. J., and M. P. Czech. 1986. Regulation of transferrin receptor expression at the cell surface by insulin-like growth factors, epidermal growth factor and platelet-derived growth factor. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:653–658.
12. Deutsch, C. J., A. Holian, S. K. Holian, R. P. Daniele, and D. F. Wilson. 1979. Transmembrane electrical and pH gradients across human erythrocytes and human peripheral lymphocytes. *J. Cell. Physiol.* 99:79–94.
13. Dunn, W. A., T. P. Connolly, and A. L. Hubbard. 1986. Receptor-mediated endocytosis of epidermal growth factor by rat hepatocytes: receptor pathway. *J. Cell Biol.* 102:24–36.
14. Dunn, W. A., and A. L. Hubbard. 1984. Receptor-mediated endocytosis of epidermal growth factor by hepatocytes in the perfused rat liver: ligand and receptor dynamics. *J. Cell Biol.* 9:2148–2159.
15. Fraker, P. J., and J. C. Speck, Jr. 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80:849–857.
16. Frøkjaer-Jensen, J. 1980. Three-dimensional organization of plasmalemmal vesicles in endothelial cells. An analysis by serial sectioning of frog mesenteric capillaries. *J. Ultrastruct. Res.* 73:9–20.
17. Garofalo, R. S., D. M. Gilligan, and B. H. Satir. 1983. Calmodulin antagonists inhibit secretion in *Paramecium*. *J. Cell Biol.* 96:1072–1081.
18. Ghitescu, L., A. Fixman, M. Simionescu, and N. Simionescu. 1986. Specific binding sites for albumin restricted to plasmalemmal vesicles of continuous capillary endothelium: receptor-mediated transcytosis. *J. Cell Biol.* 102:1304–1311.
19. Goldmacher, V. S., N. L. Tinnel, and B. C. Nelson. 1986. Evidence that pinocytosis in lymphoid cells has a low capacity. *J. Cell Biol.* 102:1312–1319.
20. Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Rusell, and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* 1:1–39.
21. Gorden, P., J. L. Carpentier, S. Cohen, and L. Orci. 1978. Epidermal growth factor: morphological demonstration of binding, internalization, and lysosomal association in human fibroblasts. *Proc. Natl. Acad. Sci. USA.* 75:5025–5029.
22. Haigler, H. T., F. R. Maxfield, M. C. Willingham, and I. Pastan. 1979. Dansylcadaverine inhibits internalization of ^{125}I-epidermal growth factor in BALB 3T3 cells. *J. Biol. Chem.* 255:1239–1241.
23. Hopkins, C. R. 1983. Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A431 cells. *Cell.* 35:321–330.
24. Hopkins, C. R., K. Miller, and J. M. Beardmore. 1985. Receptor-mediated endocytosis of transferrin and epidermal growth factor receptors: a comparison of constitutive and ligand-induced uptake. *J. Cell Sci. Suppl.* 3: 173–186.
25. Hopkins, C. R., and I. S. Trowbridge. 1983. Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. *J. Cell Biol.* 97:508–521.
26. Horisberger, M., and J. Rosset. 1977. Colloidal gold, a useful marker for transmission and scanning electron microscopy. *J. Histochem. Cytochem.* 25:295–305.
27. 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.
28. 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.
29. Larkin, J. M., W. C. Donzell, and R. G. W. Anderson. 1985. Modulation of intracellular potassium and ATP: Effects on coated pit function in fibroblasts and hepatocytes. *J. Cell. Physiol.* 124:372–378.
30. Larkin, J. M., W. C. Donzell, and R. G. W. Anderson. 1986. Potassium-dependent assembly of coated pits: new coated pits form as planar clathrin lattices. *J. Cell Biol.* 103:2619–2627.
31. Madshus, I. H., K. Sandvig, S. Olsnes, and B. van Deurs. 1987. Effect of reduced endocytosis induced by hypotonic shock and potassium depletion on the infection of Hep 2 cells by picornaviruses. *J. Cell. Physiol.* 131: 6–13.

32. Madshus, I. J., T. I. Tønnessen, S. Olsnes, and K. Sandvig. 1987. Effect of potassium depletion of Hep 2 cells on intracellular pH and on chloride uptake by anion antiport. *J. Cell. Physiol.* 131:14–22.
33. Marsh, M. 1984. Review article: the entry of enveloped viruses into cells by endocytosis. *Biochem. J.* 218:1–10.
34. Maxfield, F. R., J. Schlessinger, Y. Shechter, I. Pastan, and M. C. Willingham. 1978. Collection of insulin and α_2-macroglobulin in the same patches on the surface of cultured fibroblasts and common internalization. *Cell.* 14:805–810.
35. 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.
36. Moolenaar, W. H., L. G. J. Tertoolen, and S. W. de Laat. 1984. The regulation of cytoplasmic pH in human fibroblasts. *J. Biol. Chem.* 259: 7563–7569.
37. Moya, M., A. Dautry-Varsat, B. Goud, D. Louvard, and P. Boquet. 1985. Inhibition of coated pit formation in Hep_2 cells blocks the cytotoxicity of diphtheria toxin but not that of ricin toxin. *J. Cell Biol.* 101:548–559.
38. Olsnes, S., and K. Sandvig. 1985. Entry of polypeptide toxins into animal cells. *In* Endocytosis. I. Pastan and M. C. Willingham, editors. Plenum Publishing Corp., New York. 195–234.
39. Petersen, O. W., and B. van Deurs. 1987. Preservation of defined phenotypic traits in short-term cultured human breast carcinoma derived epithelial cells. *Cancer Res.* In press.
40. Rogers, J., T. R. Hesketh, G. A. Smith, M. A. Beaven, J. C. Metcalfe, P. Johnson, and P. B. Garland. 1983. Intracellular pH and free calcium changes in single cells using quene 1 and quin 2 probes and fluorescence microscopy. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 161:21–27.
41. Rogers, J., T. R. Hesketh, G. A. Smith, and J. C. Metcalfe. 1983. Intracellular pH of stimulated thymocytes measured with a new fluorescent indicator. *J. Biol. Chem.* 258:5994–5997.
42. Sandvig, K., and S. Olsnes. 1979. Effect of temperature on the uptake, excretion and degradation of abrin and ricin by HeLa cells. *Exp. Cell. Res.* 121:15–25.
43. Sandvig, K., and S. Olsnes. 1982. Entry of the toxic proteins abrin, modeccin, ricin and diphtheria toxin into cells. II. Effect of pH, metabolic inhibitors, and ionophores and evidence for toxin penetration from endocytotic vesicles. *J. Biol. Chem.* 257:7504–7513.
44. Sandvig, K., A. Sundan, and S. Olsnes. 1985. Effect of potassium depletion of cells on their sensitivity to diphtheria toxin and pseudomonas toxin. *J. Cell. Physiol.* 124:54–60.
45. Sandvig, K., T. I. Tønnessen, and S. Olsnes. 1987. Ability of inhibitors of glycosylation and protein synthesis to sensitize cells to abrin, ricin, shigella toxin and pseudomonas toxin. *Cancer Res.* 46:6418–6422.
46. Sandvig, K., T. I. Tønnessen, O. Sand, and S. Olsnes. 1986. Requirement of a transmembrane pH gradient for the entry of diphtheria toxin into cells at low pH. *J. Biol. Chem.* 261:11639–11644.
47. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* 51:660–672.
48. Shechter, Y. 1984. Trifluoperazine inhibits insulin action on glucose metabolism in fat cells without affecting inhibition of lipolysis. *Proc. Natl. Acad. Sci. USA.* 81:327–331.
49. Schlossman, D. M., S. L. Schmid, W. A. Braell, and J. E. Rothman. 1984. An enzyme that removes clathrin coats: purification of an uncoating ATPase. *J. Cell Biol.* 99:723–733.
50. Slot, J. W., and H. J. Geuze. 1981. Sizing of protein A-colloidal gold probes for immunoelectron microscopy. *J. Cell Biol.* 90:533–536.
51. Steinman, R. M., J. M. Silver, and Z. A. Cohn. 1974. Pinocytosis in fibroblasts. Quantitative studies in vitro. *J. Cell Biol.* 63:949–969.
52. Swanson, J. A., B. D. Yirinec, and S. C. Silverstein. 1985. Phorbol esters and horseradish peroxidase stimulate pinocytosis and redirect the flow of pinocytosed fluid in macrophages. *J. Cell Biol.* 100:851–859.
53. Thomas, J. A., R. N. Buchsbaum, A. Zimniak, and E. Racker. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry.* 18:2210–2218.
54. van Deurs, B., and K. Nilausen. 1982. Pinocytosis in mouse L-fibroblasts: ultrastructural evidence for a direct membrane shuttle between the plasma membrane and the lysosomal compartment. *J. Cell Biol.* 94:279–286.
55. van Deurs, B., Ryde Pedersen, L., A. Sundan, S. Olsnes, and K. Sandvig. 1985. Receptor-mediated endocytosis of a ricin-colloidal gold conjugate in Vero cells. Intracellular routing to vacuolar and tubulovesicular portions of the endosomal system. *Exp. Cell Res.* 159:287–304.
56. van Deurs, B., O. W. Petersen, S. Olsnes, and K. Sandvig. 1987. Delivery of internalized ricin from endosomes to cisternal Golgi elements is a discontinuous, temperature sensitive process. *Exp. Cell Res.* In press.
57. van Deurs, B., T. I. Tønnessen, O. W. Petersen, K. Sandvig, and S. Olsnes. 1986. Routing of internalized ricin and ricin conjugates to the Golgi complex. *J. Cell Biol.* 102:37–47.
58. Ward, D. McVey, and J. Kaplan. 1986. Mitogenic agents induce redistribution of transferrin receptors from internal pools to the cell surface. *Biochem. J.* 238:721–728.
59. Wileman, T., C. Harding, and P. Stahl. 1985. Receptor-mediated endocytosis. *Biochem. J.* 232:1–14.