Formation of Coated Vesicles from Coated Pits in Broken A431 Cells

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Abstract. Biochemical and morphological techniques were used to demonstrate the early steps in the endocytosis of transferrin in broken A431 cells. After binding ^{125}I-transferrin, the cells were broken by scraping and then warmed. ^{125}I-transferrin became inaccessible to exogenous anti-transferrin antibody providing a measure of the internalization process. Parallel morphological experiments using transferrin coupled to horseradish peroxidase confirmed internalization in broken cells. The process was characterized and compared with endocytosis in intact cells and showed many similar features. The system was used to show that both the appearance of new coated pits and the scission of coated pits to form coated vesicles were dependent on the addition of cytosol and ATP whereas invagination of pits was dependent on neither.

RECEPTOR-mediated endocytosis enables cells to select certain components from the surrounding medium and exclude others (Pearse and Bretscher, 1981). Ligands bind to high affinity surface receptors which are either already located in coated pits or become located in them as a result of ligand binding. Information in the cytoplasmic tail of the receptor appears responsible for incorporation into pits (Davis et al., 1986; Lazarovits and Roth, 1988) and is interpreted by coat components (100- and 50-kD accessory coat proteins) which link the receptors to the underlying clathrin coat (Robinson and Pearse, 1985; Pearse, 1985). Invagination is probably caused by rearrangement of the coat (Heuser and Evans, 1980; Pearse and Crowther, 1987) and, by generating a high surface area to volume ratio, the coated pit ensures that the volume of free fluid taken up is as small as possible. This reduces the amount of contaminating ligands taken into the cell. After scission of the deeply invaginated pit to form a coated vesicle, the coat is removed and the vesicle fuses with an endosome thereby exposing the ligand–receptor complexes to the low pH inside this organelle (Tycko and Maxfield, 1982). Some complexes are unaffected and pass on to another part of the plasma membrane as happens during transcytosis of IgA (Mostov and Simister, 1985). Other complexes are sent directly to lysosomes for degradation as happens to complexes of growth factors and their receptors (Carpenter, 1987). Yet others are dissociated, the ligands passing to lysosomes and the receptors back to the cell surface (Goldstein et al., 1985), probably by a mechanism that mirrors the uptake pathway (Geuze et al., 1984).

A variety of nutrients take this latter pathway. Iron uptake, for example, is mediated by ferri-transferrin, which binds to surface transferrin receptors and is delivered into the acidic environment of the endosome. This releases the ferric ions and the depleted carrier, apo-transferrin, recycles to the plasma membrane still bound to receptor. Here it is released allowing more ferri-transferrin to bind, thereby continuing the transferrin cycle (Octave et al., 1983; Dautry-Varsat et al., 1983; Klausner et al., 1983).

Little is known about the mechanism underlying receptor-mediated endocytosis largely because the component parts are located, for the most part, on the cytoplasmic side of the membrane and are therefore not directly accessible to biochemical manipulation. Cells have to be broken and considerable information has been obtained by analysis of isolated intermediates, especially coated vesicles (Pearse, 1987) and endosomes (Helenius et al., 1983). What has been missing to date is information as to how one intermediate is transformed into another and what additional components are needed for these steps. The classical biochemical approach to obtaining such information is to set up cell-free assays which measure one or more steps of a pathway (Buchner, 1897; Harden and Young, 1906). Using this approach, an ATPase has been purified that uncoats coated vesicles in vitro and is thought to have the same function in vivo (Rothman and Schmid, 1986). The fusion of endocytic vesicles has also been studied and a preliminary characterization of the process has been obtained (see Warren et al., 1988). In this study we have used the same approach to examine earlier steps in the endocytic pathway and have been able to identify several of the intermediates involved and the conditions required to convert one into another.

Materials and Methods

Materials

All media and supplements were obtained from Flow Laboratories, Rickmansworth, England. Tissue culture plastic was obtained from Gibco Ltd.,

Paisley, Scotland. All other reagents, unless otherwise stated, were obtained from Sigma Chemical Co. (Poole, England) or BDH Chemicals Ltd. (Poole, England).

Cells

A431 cells (a gift from Professor Colin Hopkins, Imperial College, London) were grown in DME supplemented with 10% (vol/vol) FCS, and 100 U/ml each of penicillin and streptomycin. Cells were grown at 37°C in an atmosphere of 95% air/5% CO_2, and passaged every 2–3 d at a 1:5 dilution. Cells were not used past passage 40.

Antibodies

Sheep antitransferrin antiserum, a gift from the Scottish Antibody Production Unit, was heat treated for 30 min at 56°C and stored at 4°C. The volume of sheep antitransferrin antibody used per assay was in excess of that required to immunoprecipitate 95–98% of the surface transferrin.

Rabbit anti-sheep IgG was purchased from Cooper Biomedical, Lorne Diagnostics (Bury St. Edmonds, England). *Staphylococcus aureus* cells were completely coated with rabbit anti-sheep antibody and an excess was used to collect the immune complex.

Iodinated Transferrin

Iodinated transferrin was prepared according to the iodogen method (Fraker and Speck, 1978) with the modifications described by Woodman and Warren (1988). The final preparation of transferrin had a protein concentration of 50 μg/ml and a specific activity of 4–8 × 10^6 cpm/μg. Radiolabeled transferrin was stored at 4°C for up to 2 wk.

Preparation of Broken Cells

A431 cells were broken in a manner similar to that used by Beckers et al. (1987). They were grown to near confluency (80–90%) on large Petri dishes (140-mm-diam) or large plates (243 × 243 mm). After washing three times with ice-cold Dulbecco's PBS, the cells in Petri dishes were incubated in 5 ml of binding medium, those in large plates in 10 ml of binding medium (DME supplemented with 20 mM Hepes-NaOH, pH 7.4, and 0.1% (wt/vol) BSA), containing ^{125}I-transferrin (1 μg/ml) for 10 min at 4°C with rocking. The cells were then washed four times with 100 mM KCl, 85 mM sucrose, 1 mM magnesium acetate, and 20 mM Hepes-NaOH, pH 7.4 (KSHM)[1], and drained for 2–3 min to remove as much buffer as possible without the cells drying out. Cells were removed from the dish by scraping with a section of a rubber bung with a straight edge. After gentle resuspension using a Gilson 1-ml pipette tip with the end removed, cells were used immediately. The preparation of broken cells resulted in an 8–10-fold dilution of cytosol.

Cell breakage was determined by the release of lactic dehydrogenase assayed essentially by the method of Kornberg (1955). An aliquot of the broken cell suspension was centrifuged at 1,000 g_{av} for 10 min at 4°C. Both the supernatant and pellet (after resuspension in KSHM) were assayed for lactic dehydrogenase activity in the presence of 0.5% (wt/vol) Triton X-100. The percentage of the total lactic dehydrogenase activity present in the supernatant was taken to be the measure of cell breakage.

Conditions of Incubation

Broken cell suspensions (10–50 μl) were placed in Eppendorf tubes and a one-tenth volume of an ATP-regenerating or -depleting system was added. The ATP-regenerating system comprised 1 vol 40 mM MgATP, pH 7.0, 1 vol 200 mM creatine phosphate in H_2O and 2 vol creatine phosphokinase (0.2 mg/ml in 50% glycerol). For the ATP-depleting system, hexokinase, supplied as an $(NH_4)_2SO_4$ precipitate, was pelleted and resuspended to 500 IU/ml in 100 mM glucose. The samples were warmed to 31°C and incubated for 15 min unless otherwise stated. After chilling on ice, 2 μl sheep antitransferrin antiserum in 100 μl KSHM was added and the samples left on ice for 90 min with frequent but gentle agitation. Addition of 900 μl of KSHM and mixing was followed by centrifugation at 1000 g_{av} for 10 min at 4°C. The supernatants were removed. The pellets were resuspended in 100 μl of immunoprecipitation buffer (IB; 100 mM Tris-Cl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1% [wt/vol] Triton X-100 and 1 mg/ml BSA) containing 0.5 μg of transferrin. After 5 min on ice, coated *Staphylococcus aureus* cells (10 μl of a 10% suspension in IB, coated with rabbit anti-sheep antibody) were added and the samples incubated at 37°C for 30 min. The samples were then centrifuged at low speed for 4 min at room temperature in a Microcentaur centrifuge and the pellets and supernatants counted. The loss of accessibility was expressed as the percentage of ^{125}I-transferrin in the supernatant over the total (pellet and supernatant) ^{125}I-transferrin present at zero time.

For the cytosol dependence experiments, broken cells were diluted to 1 ml with KSHM and then centrifuged at 1,000 g_{av} for 10 min at 4°C. After removal of the supernatant the pellets were resuspended either in KSHM or cytosol (10–20 mg/ml) containing an ATP-regenerating or -depleting system. The remainder of the incubation was carried out as described above.

For determination of the pH dependence of internalization the buffers used consisted of 100 mM KCl, 85 mM sucrose, 1 mM magnesium acetate and, for pH 6.0–6.8, 20 mM Mes; for pH 6.8–7.7, 20 mM Mops; and for pH 7.4–8.0, 20 mM Hepes.

Preparation of Cytosol

Confluent monolayers of A431 cells were washed four times in KSHM, scraped, and homogenized at 4°C by passing the suspension six times through a stainless steel ball bearing homogenizer with an 8-μm clearance (Balch et al., 1984). After centrifugation in a rotor (model 70.1 Ti; Beckman Instruments, Inc., Palo Alto, CA) for 1 h at 230,000 g_{av} aliquots of the supernatant were frozen and stored in liquid nitrogen. Protein concentrations were determined according to the method of Bradford (1976).

Internalization in Intact Cells

To measure internalization of ^{125}I-transferrin in intact cells the cells were grown in Petri dishes (3.5-cm-diam) and, after washing three times with PBS, were incubated with 0.6 ml of ^{125}I-transferrin (1 μg/ml). Internalized transferrin was measured using two methods. (*a*) Surface bound label was removed by incubation of the cells at 4°C in acetic acid saline (0.2 M acetic acid, 0.5 M NaCl, pH 2.4) for 10 min (Hopkins and Trowbridge, 1983). The cells were then digested with 1 M NaOH for 1 h at 4°C. (*b*) Cells were treated with 2 μl of sheep antitransferrin antibody in 200 μl KSHM. After removal of excess antibody and solubilization in IB containing 5 μg/ml unlabeled transferrin, surface bound ^{125}I-transferrin was immunoprecipitated using *Staphylococcus aureus* cells coated with rabbit anti-sheep antibody.

Transferrin Conjugated to Horseradish Peroxidase (Transferrin-HRP)

Conjugates were prepared according to the method of Bretscher and Thomson (1985). Horseradish peroxidase was extensively succinylated and then reacted with transferrin. Products were gel filtered in Sephadex G-200 in 2 mM Hepes-NaOH, pH 7.4, 150 mM NaCl to separate conjugates from free transferrin and HRP. Analysis by SDS-PAGE, showed that mono- and divalent conjugates (transferrin-HRP and transferrin-2HRP) had been formed. The conjugates were stored at −20°C at a concentration of 0.2 mg/ml.

Preparation of Samples for Morphological Analysis

Near confluent A431 cells were incubated in serum-free medium for 1 h at 37°C to remove bovine transferrin. This increased the binding of transferrin-HRP which appeared to have a lower affinity for the transferrin receptor than ^{125}I-transferrin. The cells were then washed in PBS and incubated for 2 h at 4°C in binding medium containing transferrin-HRP (4 μg/ml). The cells were washed six times in KSHM buffer at 4°C and broken as described above. The conjugate was shown to bind specifically by incubating the cells in transferrin-HRP in the presence of a 100-fold excess of unlabeled human transferrin. Under these conditions, no HRP reaction product was detected.

Aliquots (50 μl) of broken cells were mixed with 5 μl of the ATP-regenerating system and either kept on ice for 0 or 15 min, or incubated at 37°C for 5, 10, or 15 min. After incubation, 100 μl of KSHM was added and each tube was kept on ice for 90 min. The tubes were then centrifuged at 1,000 g_{av} for 10 min at 4°C and the cells resuspended in 1 ml of fixative (mixture of 1 vol of KSHM and 1 vol of 1% [wt/vol] glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2). Fixation was carried out for 1 h at room temperature. Fixed cells were washed three times in PBS, pH 7.4, and then resuspended in 1 ml of 1 mg/ml 3.3'-diaminobenzidine (tetrahydrochloride salt) and 0.3% hydrogen peroxide in PBS, pH 7.4. After incubation for 30

1. *Abbreviations used in this paper*: IB, immunoprecipitation buffer; KSHM, 100 mM KCl, 85 mM sucrose, 1 mM magnesium acetate, and 20 mM Hepes-NaOH, pH 7.4.

min at room temperature, cells were washed three times in PBS and then pelleted in a centrifuge (model J2-21; Beckman Instruments, Inc., Palo Alto, CA) at 7,100 g_{av} for 10 min so that flat pellets were obtained.

The pellets were postfixed in 1% (wt/vol) osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, dehydrated in a series of graded solutions of ethanol and embedded intact in Epon 812 resin (Taab Laboratories, Berkshire, UK).

For studies on the ATP and cytosol dependence aliquots of broken cells were first diluted to 1 ml in KSHM, centrifuged, and then resuspended in KSHM or cytosol with an ATP-regenerating or -depleting system. They were incubated for 15 min at 0 or 31°C and then processed as described above.

To make serial sections each pellet was sectioned in the periphery, perpendicular to the bottom of the pellet. A pyramid was trimmed at a random location along the profile of the pellet in such a way that each section contained cells from both the top and bottom of the pellet. Serial sections were cut using a Reichert-Jung ultramicrotome and mounted on carbon/formvar-coated slot grids. The average section thickness was 30 nm (coefficient of error: 7%). Sections were stained for 15 min with a 3% (wt/vol) solution of uranyl acetate and for 5 min with a solution of lead citrate according to the method of Reynolds (1963) and viewed at 60 kV in a Jeol 1200 EX electron microscope.

Morphological Characterization of Broken and Intact Cells

BSA conjugated to 6-nm gold particles (BSA-gold) was dialysed against KSHM and 1 vol was then incubated with 1 vol of broken cells at 0°C for 5 min. BSA-gold was prepared according to the method cited in Roth (1983) and was a gift from Dawn Wood (Biochemistry Department, University of Dundee, Scotland). The cells were fixed, postfixed, and processed as described above.

When viewed in the electron microscope dark cells did not contain gold and were therefore classified as intact. Cells with a light appearance always contained gold particles and were therefore classified as broken. However, they did not always exhibit holes in the profiles examined. Intact and broken cells were identified in all subsequent experiments according to the appearance of the cytoplasm.

Morphological Quantitation

The numbers of labeled coated pits and coated vesicles per nucleus in broken cells were obtained using the stereological principle called the "disector" (Sterio, 1984). Particles are counted if they are sampled by a two-dimensional unbiased sampling frame and do not intersect a parallel "look-up" plane with a known separation from the first plane. The number (Q−) of particles that have disappeared in the look-up gives an estimate of the total number of particles (N) inside the reference space (the pellet in our case). By dividing the estimate of the number of coated pits and vesicles (n) in the reference space by the same estimate for nuclei (N), we were able to calculate the numbers of coated structures per nucleus using the following formula:

$$n/N = \Sigma q- \times V(\text{dis})/\ \Sigma Q- \times v(\text{dis}), \quad (1)$$

where $\Sigma q-$ refers to the disectors for coated pits, coated vesicles, and uncoated structures; $\Sigma Q-$ refers to the nuclei disectors; v(dis) is the volume of the disector used to count coated and uncoated structures; and V(dis) is the volume of the disector used to count nuclei. In Eq. 1,

$$V(\text{dis}) = A \times K \times T, \quad (2)$$

where A is the surface of the sampling frame, K is the number of sections between the first plane and the look-up, and T is the section thickness. If both estimates are obtained from the same stack of serial sections, the numbers of coated structures per nucleus are given by the following formula:

$$n/N = \Sigma q- \times \mathrm{A} \times \mathrm{K} / \Sigma Q- \times a \times k, \quad (3)$$

where A and K refer to nuclei disectors and a and k refer to disectors for coated and uncoated structures. T is cancelled in Eq. 3, which means that there is no need to measure the section thickness (Gundersen, 1986).

Disectors To Count Nuclei. Two sections with an average separation of 76 sections (corresponding to 2.3 μm) were sampled from each pellet. The first and the last section were used in turn as the look-up. This yields twice as much information from the same series of sections. Only nuclei from broken cells were counted. Counting was carried out on micrographs taken at 200× and printed with a three- to fourfold higher magnification. The average number of nuclei counted per disector was 25.

Disectors To Count Coated Pits, Coated Vesicles, and Uncoated Structures. Four disectors were placed within the stack of sections used to count the nuclei. They were placed across the sections systematically with a random start, and were placed on different sets of sections each time. Each disector was composed of three consecutive serial sections, the first and third sections being used in turn as the look-up. Counting was carried out at the electron microscope or using micrographs. In the early experiments, the frame on the viewing screen of the electron microscope was used as a sampling frame and its surface area was 316 μm^2 (real size). In the later experiments, counting was carried out on micrographs taken at a magnification of 3,000 and printed with a further 3.5-fold higher magnification, and the sampling frame was chosen to have the same surface area of 316 μm^2.

Measurement of the Degree of Invagination of Coated Pits. A representative sample of the coated pits was measured as described previously (Pypaert et al., 1987) except that the ratio of the maximal width of the neck to the maximal coat length was used as an index of invagination. These two measurements were sometimes measured on different sections through the same pit. Coated pits were photographed at 25,000× and printed with a sixfold higher magnification before being measured. The coated pits were placed in one of three categories as defined in Results. The percentage in each category was then used to estimate the total number per nucleus in each category.

Results

Internalization of ^{125}I-Transferrin in Broken Cells

^{125}I-Transferrin was bound at 4°C to A431 cells which were then broken by scraping. After warming to allow internalization they were chilled on ice and excess sheep antitransferrin antibody was added to bind all of the remaining surface-bound transferrin. Internalized transferrin should be protected. After removal of unbound antibody, by centrifugation, the resuspended cells were treated with excess unlabeled transferrin to mask any remaining antibody binding sites. At the same time the cells were solubilized with detergent and incubated with *Staphylococcus aureus* cells coated with rabbit anti-sheep antibody. The immune complexes of surface transferrin became bound and were removed by centrifugation. ^{125}I-transferrin remaining in the supernatant represented the amount internalized during the incubation.

Examining the transferrin cycle in A431 cells offers a number of advantages for these measurements. There are a large number (10^5) of transferrin binding sites on the cell surface (Hopkins and Trowbridge, 1983) and most of these are in

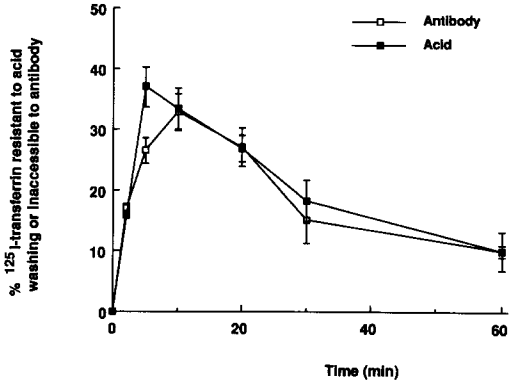


Figure 1. Endocytosis of ^{125}I-transferrin in intact A431 cells. Near confluent A431 cells were incubated with ^{125}I-transferrin and warmed at 31°C for various times. After quenching on ice, surface ^{125}I-transferrin was either removed using acid or bound by antibody. Values are expressed as the means of three separate experiments ± SEM, each performed in duplicate.

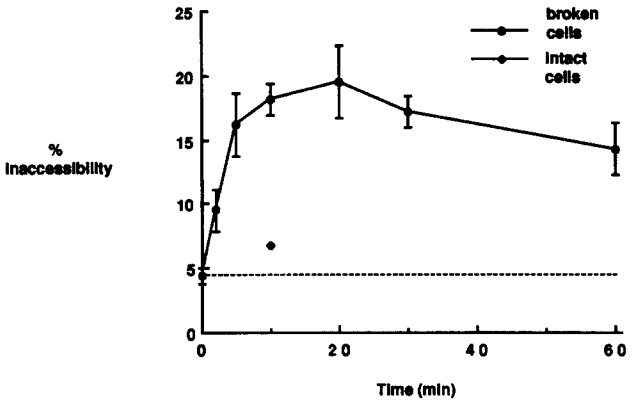


Figure 2. Internalization of ^{125}I-transferrin in broken A431 cells. Broken cell preparations were warmed in the presence of ATP at 31°C for various times. The amount of internalized ^{125}I-transferrin was measured by inaccessibility to immunoprecipitation. The single point represents the signal calculated to be due to the remaining intact cells in the preparation. The dotted line represents the percentage ^{125}I-transferrin inaccessible to antibody in intact and broken cells held at 0°C. Values are expressed as the means of three separate experiments ± SEM, each performed in triplicate.

coated pits (Hopkins, 1985) which contributes to the rapid uptake seen in these cells. They are easily broken by scraping without extensive damage to their intracellular organization (see Fig. 3 *a*). In addition transferrin can be iodinated to a high specific activity which makes the assay very sensitive.

To check that inaccessibility to antibody provided an accurate measure of internalization, intact A431 cells were used to compare this method with the standard acid-washing procedure (Hopkins and Trowbridge, 1983) for the removal of cell surface ligand. Fig. 1 shows that both methods gave very similar results. Internalization was rapid, peaking at 5–10 min, followed by a drop in intracellular transferrin previously shown to represent recycling to the plasma membrane (Hopkins and Trowbridge, 1983; Ciechanover et al., 1983).

Internalization in broken cells was then measured in the presence of ATP (Fig. 2) and the rate was found to be similar to that in intact cells. The peak occurred slightly later, at 20 min, and the fall was not as dramatic suggesting that recycling might not be occurring in this system. At the peak, ~15% of the transferrin became inaccessible compared with just under 40% in intact cells (cf. Figs. 1 and 2).

The assay cannot discriminate between broken and intact cells so it was important to show that most of the cells were broken by scraping in each experiment. The extent of cell breakage, as measured by the release of the cytosolic marker lactic dehydrogenase, was in all cases >90%. Even allowing for the possibility that the remaining intact cells function as well in the assay as do intact cells in the monolayer (Fig. 1), their contribution to the observed internalization cannot account for the size of the signal seen (Fig. 2).

An estimate of the number of broken cells was also obtained by examining their appearance by electron microscopy (Fig. 3 *a*). In a separate series of experiments using BSA-gold (see Materials and Methods) it was shown that cells with a dark appearance were intact whereas those with a light appearance were broken. Using this criterion, <10% of the cells remained intact after scraping. To eliminate the possibility that resealing of the broken cell membranes occurred during the course of the assay, cells were warmed to 37°C for 15 min, chilled for 5 min, and then incubated with BSA-gold. All of the cells classified by their appearance as open were labeled with BSA-gold (Fig. 3 *c*) showing that they had not resealed during the course of the incubation. The same result was obtained with samples kept at 0°C (Fig. 3 *b*). Resealed cells were not therefore contributing to the signal observed.

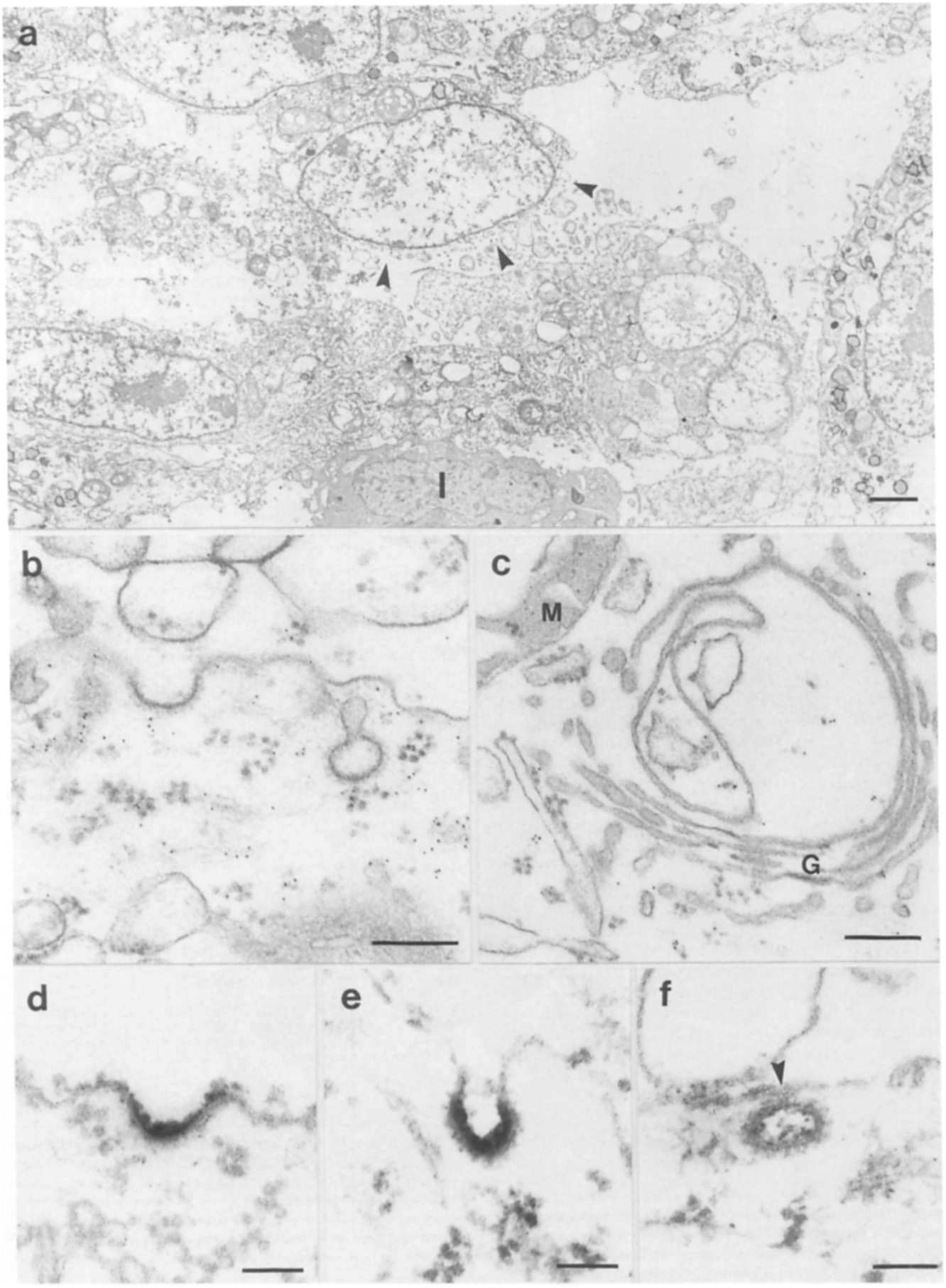
A number of other controls were carried out (Table I). Addition of ^{125}I-transferrin to broken cells to which unlabeled transferrin had been prebound gave only 12% of the normal signal showing that the assay measures a receptor-mediated event. Addition of detergent at the same time as the antitransferin antibody also abolished the signal showing that the inaccessible transferrin is within a detergent-sensitive space likely to be a vesicle. The inaccessible transferrin was also not simply transferrin that had undergone a conformational change such that it no longer bound antibody. Addition of more antibody and coated *Staphylococcus aureus* cells abolished the signal. Finally both *Staphylococcus aureus* cells and antibody were responsible for the separation of two

Table I. Validation of the Assay

Condition	Protected ^{125}I-transferrin
	% of control
Control	100.0 ± 5.3
Cells pretreated with excess transferrin before adding ^{125}I-transferrin	12.4 ± 1.4
Triton X-100 added at same time as antibody	8.7 ± 3.3
^{125}I-Transferrin in supernatant treated with more antibody and *Staphylococcus aureus* cells	2.1 ± 0.2
Staphylococcus aureus cells omitted	900.0 ± 5.9
Sheep anti-transferrin antibody omitted	925.0 ± 3.3

Scraped cells were incubated for 15 min at 31°C and treated as indicated. The numbers are expressed as a percentage of the amount of ^{125}I-transferrin protected in a control assay. Values represent the mean ± SEM for three experiments, each of which was carried out in duplicate.

Figure 3. Electron microscopy of scraped A431 cells. (*a*) Unlabeled; (*b* and *c*) BSA-gold; (*d–f*) transferrin-HRP. (*a*) Low magnification view of A431 cells fixed immediately after scraping. The contrast was enhanced by staining with tannic acid before dehydration (Pypaert et al., 1987). The arrowheads point to portions of a cell from which the plasma membrane has been removed. Note the presence of an intact cell (*I*) showing darker cytoplasm. (*b*) Broken cell incubated with BSA-gold (6-nm particles) at 0°C. (*c*) Broken cell incubated with BSA-gold after incubation at 37°C for 15 min. *G*, Golgi stack; *M*, mitochondrion. (*d*) Example of a shallow coated pit labeled with transferrin-HRP. (*e*) Deeply invaginated labeled coated pit with a neck diameter equal to one-fifth of the coat length. (*f*) Unclassified labeled coated pit. A connection to the plasma membrane can be seen (*arrowhead*) although the two membrane bilayers of the neck are not clearly defined. Bars: (*a*) 2 μm; (*b* and *c*) 200 nm; (*d–f*) 100 nm.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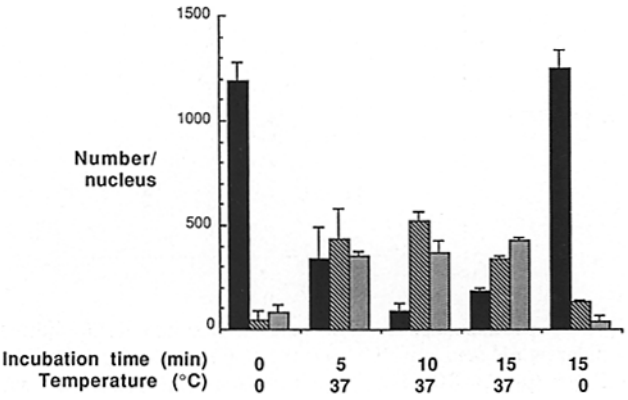

Figure 4. Time course of invagination of coated pits in broken cells. Cells, labeled with transferrin-HRP at the surface, were scraped and incubated at 0 or 37°C for varying times. The number of labeled coated pits in each category was estimated. Values are expressed as the mean of three separate experiments ± SEM. ■, shallow coated pits; ▧, deeply invaginated coated pits; ▨, unclassified coated pits.

populations of transferrin. In the absence of one or the other almost all of the ^{125}I-transferrin remained in the supernatant.

Internalization of Transferrin-HRP in Broken Cells

Invagination of coated pits was first examined in broken cells warmed for varying times. Cells were incubated with transferrin-HRP (4 μg/ml) before scraping and then processed for electron microscopy. Broken cells were identified by the light appearance of their cytoplasm (see Materials and Methods). Between 80 and 90% of the coated pits were labeled and, unless otherwise stated, the results refer only to labeled structures. The number of coated pits was estimated from serial sections using the disector method which samples particles with equal probability irrespective of size. This is important in the case of coated pits because their sizes vary with invagination and, if counted on single sections, the estimates could be biased towards less invaginated pits that have a greater chance of being sampled. This method also allows the estimate of the number of pits and vesicles per cell to be obtained without the need to section entire cells. The results are expressed as number/nucleus which is equivalent to the number/cell assuming that each cell has only one nucleus.

Coated pits were placed in one of three categories: (*a*) shallow, (*b*) deeply invaginated, and (*c*) unclassified. The profiles of shallow coated pits had neck diameters that were greater than one-fifth of the coat length (Fig. 3 *d*). The deeply invaginated coated pits (Fig. 3 *e*) had neck diameters equal to or less than one-fifth of the coat length. Unclassified pits had necks that could not be measured because the two membrane bilayers were not clearly discernible (Fig. 3 *f*). However, qualitative examination showed that most were deeply invaginated pits rather than shallow ones.

The results are presented in Fig. 4. The number of shallow pits fell by 72% in just 5 min and remained low for the rest of the incubation. This fall was matched by a corresponding

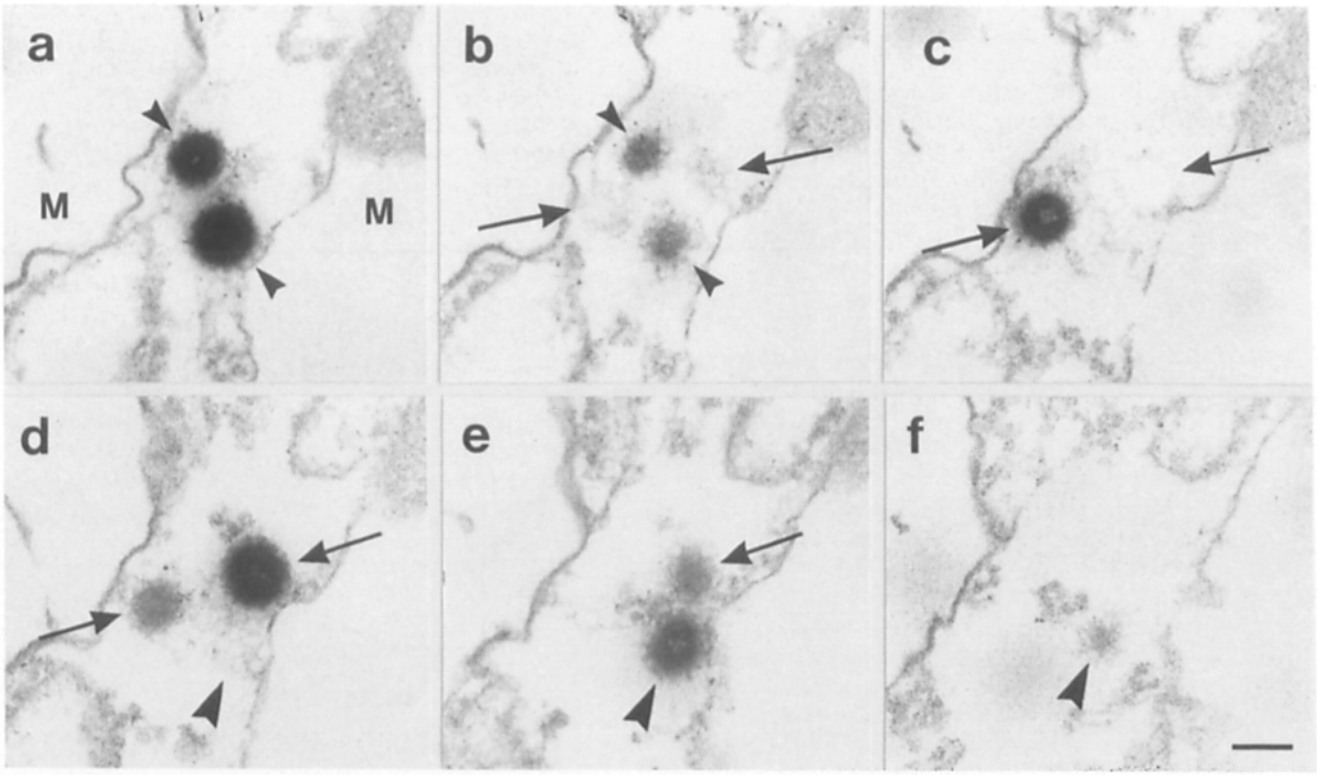

Figure 5. Series showing labeled coated vesicles. Series of six consecutive sections (*a–f*) showing labeled coated vesicles formed in a broken cell after incubation at 37°C for 10 min. Two new vesicles (*arrows*) appear in *b*, one of which is still present in *e* but neither are in *f*. The two vesicles in *a* (*small arrowheads*) are disappearing in *b*. A fifth vesicle (*large arrowhead*) appears in *d* and is present in the next two sections. The vesicles are close to two mitochondria (*M*), which show a dilated intermembrane space, as seen at low magnification. Bar, 100 nm.

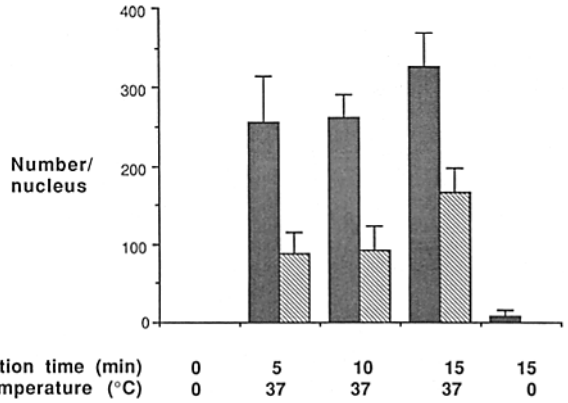

Figure 6. Appearance of labeled coated vesicles and uncoated structures in broken cells. Cells, labeled with transferrin-HRP at the surface, were scraped and incubated at 0 or 37°C for varying times. The number of labeled coated vesicles and uncoated structures was estimated. Values are expressed as the mean of three separate experiments ± SEM. ▨, coated vesicles; ■, uncoated structures.

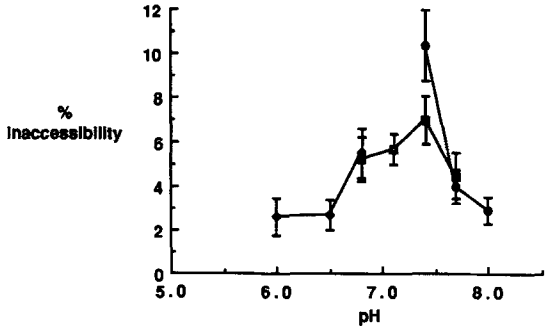

Figure 8. pH dependence. Cells were scraped into KSM adjusted to different pH values with Mes (pH 6.0–6.8), Mops (pH 6.8–7.7), or Hepes (pH 7.4–8.0). They were incubated at 31°C for 15 min and the extent of internalization measured. Values are expressed as the mean of three experiments ± SEM, each performed in duplicate. ◆, Mes; ■, Mops; ●, Hepes.

rise in the number of deeply invaginated and unclassified pits. Invagination did not occur at 0°C over the 15-min period.

In the same experiment the scission of coated pits to form coated vesicles was examined. Coated vesicles were defined as coated structures with no visible connection to the plasma membrane in serial sections (Fig. 5). Given the section thickness (30 nm) compared to the thickness of two adjacent bilayers (15 nm) the chance of missing a neck was small. The results, shown in Fig. 6, demonstrate the rapid formation of coated vesicles in broken cells. At zero time there were no coated vesicles containing transferrin-HRP, whereas at 5 min, there were ~250 per nucleus, a number which increased slightly during the succeeding 10 min. Over the same time period there was an increase in the number of labeled uncoated structures. These appeared as uncoated vesicles and tubules which may represent endosomes, the next stage on the endocytic pathway. Essentially no coated vesicles nor uncoated structures were formed at 0°C at least over the 15-min time period.

Comparison of Endocytosis in Intact and Broken Cells

Endocytosis is very sensitive to temperature (Marsh et al., 1983) and, as shown in Fig. 7, intact A431 cells do not internalize transferrin <10°C. Above this temperature the rate doubles every 5–10°C. Broken cells gave very similar results (Fig. 7).

Internalization in broken cells was also sensitive to changes in pH (Fig. 8) with peak internalization at pH 7.4, close to the physiological pH (see Hesketh et al., 1985). The decrease observed below pH 7.4 compares well with the known inhibition of endocytosis in intact cells (Sandvig et al., 1987).

Cycloheximide, chloroquine, monensin, cytochalasin B, and nocodazole do not affect endocytosis in intact cells (Anderson et al., 1982; Marsh and Helenius, 1980; Helenius et al., 1980). These drugs were also found to have no effect on internalization in broken cells measured after 10 min at 31°C (data not shown).

ATP Dependence of Internalization

Endocytosis is known to be affected by energy inhibitors in intact cells (Marsh and Helenius, 1980; Dautry-Varsat et al., 1983) so broken A431 cells were incubated in the presence or absence of ATP. As shown in Fig. 9, the time course was the same but the signal in the presence of ATP was almost double. In the absence of ATP the signal was greater than that which could be accounted for by the intact cells in the preparation.

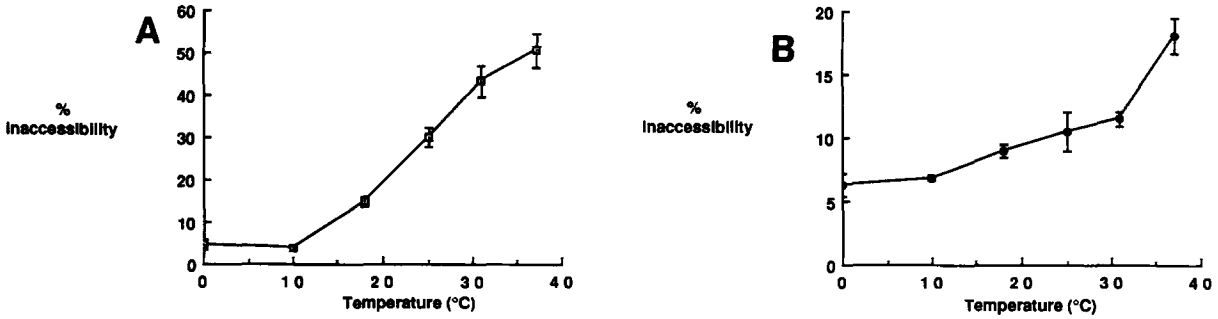

Figure 7. Temperature dependence. Intact (*A*) and broken (*B*) cells, both with bound ^{125}I-transferrin, were incubated at various temperatures for 5 min. The extent of internalization was measured in both cases and the results adjusted to account for differences in cell number. Internalization is expressed as a percentage of ^{125}I-transferrin present at zero time. Values are expressed as the means of three experiments ± SEM, each performed in triplicate. *A*, intact cells; *B*, scraped cells.

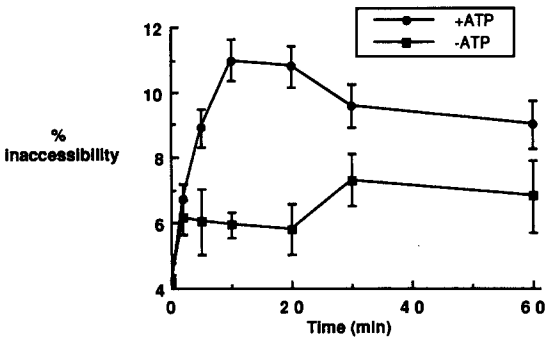

Figure 9. ATP dependence. Broken cells were incubated at 31°C for varying times in the presence of an ATP-regenerating or ATP-depleting system and the extent of internalization measured. Values are expressed as the means of three separate experiments ± SEM, each performed in duplicate.

Nonhydrolyzable analogues of ATP added in the presence of ATP inhibited internalization (Fig. 10). This shows that ATP hydrolysis is required.

Requirement for Cytosol

Broken cells were pelleted and resuspended in either buffer or cytosol, in the presence or absence of ATP, and the amount of inaccessible ^{125}I-transferrin was measured after 15 min at 31°C. As shown in Fig. 11, both ATP and cytosol were needed for maximal internalization though the percentage inaccessibility was slightly lower than that in the original broken cells. This was probably because of damage caused by the washing and centrifugation procedures. In the absence of either ATP or cytosol, internalization was lower but still significant whereas in the absence of both components the signal was no greater than that which could be accounted for by the intact cells in the preparation. The absence of a signal in this latter case was not simply due to irreparable damage to the broken and washed cells because subsequent addition of both components restored internalization to near normal levels (data not shown).

A parallel morphological study was carried out using transferrin-HRP. The number of labeled coated pits, coated vesicles, and uncoated structures was counted as was the number of unlabeled coated pits. Table II shows the results of two experiments both of which show a two- to threefold increase in the number of coated pits in the presence of added cytosol and ATP but only after warming to 31°C. This increase was significantly different from most of the other incubations. The numbers of coated pits in the absence of ATP or cytosol or both were approximately the same and not significantly different from each other.

The formation of labeled coated vesicles and uncoated structures was only observed in the presence of both ATP and cytosol and after warming to 31°C (Table II).

The invagination of labeled coated pits was not dependent on the presence of ATP or cytosol. As shown in Fig. 12 the number of shallow pits in each incubation decreased after warming at 31°C with a concomitant increase in the number of deeply invaginated pits and unclassified pits. At 0°C invagination did not occur.

Discussion

The formation of coated pits, their invagination, and subsequent scission to form coated vesicles are three of the early steps in the pathway of receptor-mediated endocytosis. We have demonstrated each of these in broken A431 cells using a combination of biochemical and morphological techniques. The biochemical technique measured the loss of accessibility of internalized ^{125}I-transferrin to antibody. The morphological technique used transferrin-HRP to label the different intermediates and their numbers were counted using the disector method.

The evidence that endocytosis was occurring can be summarized as follows. First, the kinetics of internalization of ^{125}I-transferrin were very similar in broken and intact cells (Figs. 1 and 2). The initial rate was rapid in both cases rising sharply up to 5 min. The only significant differences were that peak internalization occurred slightly later in broken cells and recycling was either reduced or absent. Second, en-

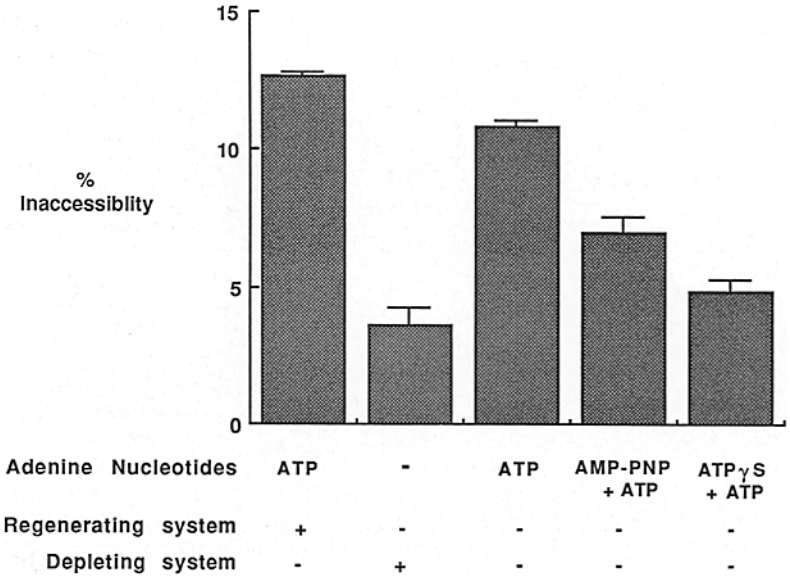

Figure 10. Effect of ATP analogues. Broken A431 cells were incubated with either an ATP-regenerating system; an ATP-depleting system; 1 mM ATP; 1 mM ATP/1 mM AMP-PNP; or 1 mM ATP/1 mM ATPγS. The amount of internalization was measured after 15 min at 31°C. Values are expressed as the means of three separate experiments ± SEM, each performed in triplicate.

Table II. Effect of Added ATP and Cytosol on the Numbers of Endocytic Intermediates in Broken Cells

	Number per nucleus							
	31°C				0°C			
	+ cytosol/ + ATP	+ cytosol/ − ATP	− cytosol/ + ATP	− cytosol/ − ATP	+ cytosol/ + ATP	+ cytosol/ − ATP	− cytosol/ + ATP	− cytosol/ − ATP
Experiment 1								
Labeled coated pits	1663 ± 632	698 ± 295	602 ± 227	370 ± 53*	600 ± 201	592 ± 234	581 ± 171*	416 ± 168*
Total coated pits	3067 ± 377	1032 ± 466*	1381 ± 254*	995 ± 70*	1489 ± 412*	898 ± 355*	1191 ± 381*	1331 ± 97*
Labeled coated vesicles	370 ± 176	0‡	0‡	0‡	0‡	0‡	0‡	0‡
Labeled uncoated structures	185 ± 71	0‡	0‡	0‡	0‡	0‡	0‡	0‡
Experiment 2								
Labeled coated pits	1418 ± 323	385 ± 155*	359 ± 94*	444 ± 286*	576 ± 264	574 ± 341*	531 ± 160*	399 ± 190*
Total coated pits	2954 ± 671	1504 ± 465	887 ± 108*	1492 ± 467	1290 ± 481*	1634 ± 1010	1545 ± 151	1117 ± 306
Labeled coated vesicles	177 ± 76	0‡	0‡	0‡	0‡	0‡	0‡	0‡
Labeled uncoated structures	59 ± 34	0‡	0‡	0‡	0‡	0‡	0‡	0‡

Labeling efficiency was 40–50%. Values are the mean of estimates from four disectors ± SEM. The Mann-Whitney U test (Siegel, 1956) was used to compare the number of coated pits counted at 31°C in the presence of ATP and cytosol with the numbers counted under any of the other conditions.
* Comparisons carried out where $p \leq 0.05$.
‡ No coated vesicles observed.

docytosis in broken cells was dependent on temperature with little internalization below 10°C, as was the case for intact cells (Fig. 7). Third, endocytosis in broken cells was optimal at physiological pH. The decreased activity below this pH is in agreement with the known sensitivity of endocytosis in intact cells (Sandvig et al., 1987). We know of no study that examines the effect of elevated pH in intact cells but from our results we would predict that endocytosis would be inhibited. Fourth, the morphological studies show that internalization in broken cells progressed through those intermediates seen in intact cells. On warming, there was a dramatic reduction in the number of shallow pits which was accompanied by an increase in the number of deeply invaginated pits and by the appearance of labeled coated vesicles. There was also an increase in the number of uncoated structures that might represent delivery of transferrin-HRP to the next compartment on the endocytic pathway, endosomes. It is also worth noting that there was no significant loss of labeled structures over the time course of the experiments. The total number of coated pits and vesicles, after warming, differed little from that at zero time. This not only suggests that one intermediate is being converted into another but, since the broken cells were washed during the experiment, shows that the inter-

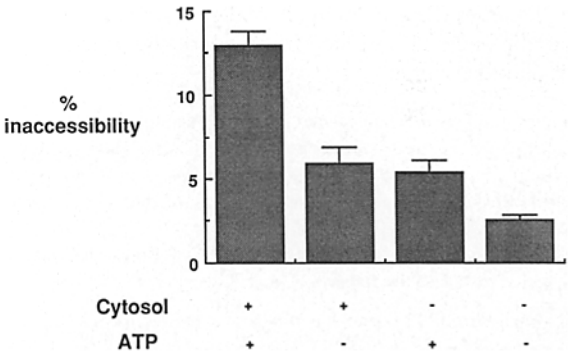

Figure 11. Cytosol dependence. After dilution and centrifugation to remove cytosol, broken cells were incubated for 15 min at 31°C with either ATP and added cytosol; an ATP-depleting system and added cytosol; ATP and KSHM buffer; or an ATP-depleting system and KSHM buffer. The internalization of ^{125}I-transferrin was measured. Values are expressed as the means of three experiments ± SEM, each performed in triplicate.

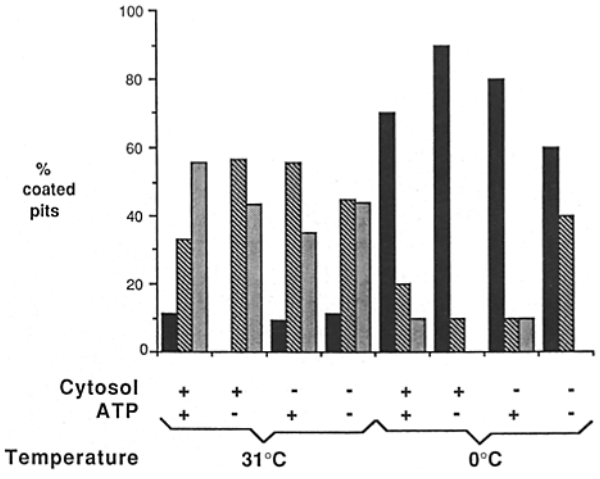

Figure 12. ATP and cytosol dependence. After dilution and centrifugation to remove cytosol, broken cells with bound transferrin-HRP were incubated at 0 or 31°C for 15 min with either ATP and added cytosol; an ATP-depleting system and added cytosol; ATP and KSHM buffer; or an ATP-depleting system and KSHM buffer. The percentage of coated pits in each of the three categories was estimated. Values are from one experiment (Experiment 1 in Table II). ■, shadow coated pits; ▩, deeply invaginated coated pits; ■, unclassified coated pits.

mediates were not free to leave the cytoplasm despite the absence of large parts of the plasma membrane. Although this might be artifactual it could be that some intermediates of the endocytic process are associated with the cytoskeleton of the cell and this association is important for some aspects of the process. In this context it is worth noting that extensive homogenization of the cells significantly reduces the signal obtained over the scraping procedure (data not shown).

By manipulating the levels of ATP and cytosol it has also proved possible to identify early intermediates and to define the broad requirements for the steps that link them. Invagination of shallow coated pits to form deeply invaginated ones was found to occur at the same rate and with the same efficiency after removal of both cytosol and ATP (Fig. 12). We cannot eliminate the possibility that residual cytosol or bound nucleotides are required for the invagination process. However >99% of the cytosol was removed in these experiments so the cells were exposed to much reduced concentrations of cytosol.

New coated pits appeared when both ATP and cytosol were added to the washed, broken cells. The increase was two- to threefold (Table II) and was not simply the consequence of redistributing the existing coat components amongst more coated pits because the average size of the coat profiles was not changed (data not shown). The coat components could have come from bound coat units too small to see by electron microscopy but this would mean that 50–70% of the coat would exist at any one time outside the coated pit. There is no evidence for this and it is far more likely that the coat components are coming from the added cytosol. Since new coated pits appear it is simplest to think that the coat components are binding to the plasma membrane and then find each other by lateral diffusion forming a new coated pit. However neither of these processes requires energy or is sensitive to temperature as borne out by the in vitro studies of Moore et al. (1987) yet the appearance of new coated pits requires exogenous ATP and warming (Table II). The role of ATP may be to do with the nucleation process but more experiments will be needed to show this. All that can be said at the moment is that the appearance of a new coated pit and its invagination are distinct, the former but not the latter requiring ATP.

Perhaps the most striking result to emerge from these studies is the requirement for both ATP and cytosol for coated vesicle formation because, in the absence of one or both of these components, it is only deeply invaginated coated pits that are formed (Table II). This argues that the scission process is the consequence of an enzymic reaction, perhaps the action of a scission ATPase. There is one earlier report that such an enzyme might exist. Kosaka and Ikeda (1983) showed that scission is inhibited in the temperature sensitive *shibire* mutant of *Drosophila melanogaster*. At the nonpermissive temperature the mutant accumulated coated pits and there was a sharp decrease in the number of coated vesicles formed at least by qualitative observation. Our system opens up the possibility of characterizing the molecular basis of this process.

The biochemical assay of internalization has been shown to be efficient with a maximal uptake of ^{125}I-transferrin of 10–20% as compared with 40–50% in intact cells (Figs. 1 and 2). Furthermore the time course of internalization using transferrin-HRP (Fig. 4 and 6) shows that a minimum of 20–30% of the labeled pits become internalized, assuming that one labeled coated vesicle gives rise to one labeled uncoated structure. This is similar to the biochemical results and suggests that the biochemical assay, under these conditions, is measuring coated vesicle formation. However, an apparent anomaly is that in the absence of either ATP or cytosol, inaccessibility of ^{125}I-transferrin is significant (Fig. 11) yet no vesicles are formed (Table II). One possibility is that before scission occurs a deeply invaginated pit is formed, the neck of which is too narrow to admit antibody. Normally this would represent a transient intermediate but under conditions where scission is inhibited it will accumulate and will be detected biochemically. Experiments to test this hypothesis are being carried out.

Coated vesicle formation in broken cells appears to be relatively slow (~60 per min) compared with the rapid rate (~2,000 per min) demonstrated in intact cells (Marsh and Helenius, 1980). In contrast invagination appears to be very fast with 72% of the shallow pits becoming deeply invaginated after 5 min. The efficiency of coated vesicle formation is also low with only 20–30% of the labeled coated pits forming coated vesicles or uncoated structures (Fig. 6 and Table II). The concentration of cytosol used in the cytosol dependence experiments was 10-fold greater than that which was present for the time course experiments yet there was no apparent increase in efficiency. Perhaps some membrane bound component(s), required for scission, may be limiting in broken cells. Fig. 6 also shows that the numbers of labeled coated vesicles and uncoated structures do not change substantially between 5 and 15 min incubation at 37°C. This suggests that the processes of uncoating and delivery to endosomes which occur rapidly in vivo (Helenius et al., 1983) are inhibited in this system.

Finally, it is worth emphasizing the importance of combining biochemical and morphological techniques when studying complex cellular processes such as endocytosis. Biochemical assays are rapid and can be used to screen different ligands and environments for their effect on the process of interest, in this case the endocytosis of transferrin. Eventually they should allow isolation and characterization of the molecules involved. Qualitative and quantitative microscopy show what the assays are measuring and allow intermediates, generated by biochemical manipulation, to be identified and studied. Together these techniques will give considerable insight into the molecules needed in the endocytic process and the role played by them.

We are grateful to Dawn Wood for the gift of BSA-gold. We would like to acknowledge the 11th European ISS Sterology Course (Martin Mere) 1987 for useful discussions on the disector method. We would also like to thank Pamela Reid and Philip Woodman for critical reading of the manuscript.

This work was supported by grants from the Medical Research Council, the Wellcome Trust, and the Imperial Cancer Research Fund.

Received for publication 13 September 1988 and in revised form 2 November 1988.

References

Anderson, R. G. W., M. S. Brown, U. Beisiegel, and J. L. Goldstein. 1982. Surface distribution and recycling of the low density lipoprotein receptor as visualized with antireceptor antibodies. *J. Cell Biol.* 93:523–531.

Balch, W. E., W. G. Dunphy, W. A. Braell, and J. E. Rothman. 1984. Reconstitution of the transport of protein between successive compartments of the

Golgi measured by the coupled incorporation of N-acetylglucosamine. *Cell.* 39:405–416.
Beckers, C. J. M., D. S. Keller, and W. E. Balch. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell.* 50:523–534.
Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72:248–254.
Bretscher, M. S., and J. N. Thomson. 1985. The morphology of endosomes in giant HeLa cells. *Eur. J. Cell Biol.* 37:78–80.
Buchner, E. 1897. Alkoholische gährung ohne hefezellen. Ber. Dtsch. Chem. Ges. 30:117–124.
Carpenter, G. 1987. Receptors for epidermal growth factor and other polypeptide mitogens. *Annu. Rev. Biochem.* 56:881–914.
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.
Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin receptors during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 80:2258–2262.
Davis, C. G., M. A. Lehrman, D. W. Russell, R. G. W. Anderson, M. S. Brown, and J. L. Goldstein. 1986. The J. D. mutation in familial hypercholesterolemia: amino acid substitution in cytoplasmic domain impedes internalization of LDL receptors. *Cell.* 45:15–24.
Fraker, P. J., and J. C. Speck. 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamine, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80:849–857.
Geuze, H. J., J. W. Slot, G. J. A. M. Strous, J. Peppard, K. von Figura, A. Hasilik, and A. L. Schwartz. 1984. Intracellular receptor sorting during endocytosis: comparative immunoelectron microscopy of multiple receptors in rat liver. *Cell.* 37:195–204.
Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russell, and W. J. Schneider. 1985. Receptor-mediated endocytosis. *Annu. Rev. Cell Biol.* 1:1–40.
Gundersen, H. J. G. 1986. Stereology of arbitrary particles. *J. Microsc.* 143:3–45.
Harden, A., and W. J. Young. 1906. The alcoholic ferment of yeast juice. *Proc. R. Soc. (Lond) B Biol. Sci.* 77:405–420.
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.
Helenius, A., I. Mellman, D. Wall, and A. Hubbard. 1983. Endosomes. *Trends Biochem. Sci.* 8:245–250.
Hesketh, T. R., J. P. Moore, J. D. H. Morris, M. V. Taylor, J. Rogers, G. A. Smith, and J. C. Metcalfe. 1985. A common sequence of calcium and pH signals in the mitogenic stimulation of eukaryotic cells. *Nature (Lond.).* 313:481–484.
Heuser, J., and L. Evans. 1980. Three-dimensional visualization of coated vesicle formation in fibroblasts. *J. Cell Biol.* 84:560–583.
Hopkins, C. R. 1985. The appearance and internalization of transferrin receptors at the margins of spreading human tumour cells. *Cell.* 40:199–208.
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.
Klausner, R., G. Ashwell, J. Van Renswoude, J. B. Harford, and K. R. Bridges. 1983. Binding of apotransferrin to K562 cells: explanation of the transferrin cycle. *Proc. Natl. Acad. Sci. USA.* 80:2263–2266.
Kornberg, A. 1955. Lactic dehydrogenase of muscle. *Methods Enzymol.* 1:441–454.
Kosaka, T., and K. Ikeda. 1983. Reversible blockage of membrane retrieval and endocytosis in the garland cell of the temperature-sensitive mutant of *Drosophila melanogaster, shibire. J. Cell Biol.* 97:499–507.
Lazarovits, J., and M. Roth. 1988. A single amino acid change in the cytoplasmic domain allows the influenza virus hemagglutinin to be endocytosed through coated pits. *Cell.* 53:743–752.
Marsh, M., and A. Helenius. 1980. Adsorptive endocytosis of Semliki Forest virus. *J. Mol. Biol.* 142:439–454.
Marsh, M., E. Bolzau, and A. Helenius. 1983. Penetration of Semliki Forest virus from acidic prelysosomal vacuoles. *Cell.* 32:931–940.
Moore, M. S., D. T. Mahaffey, F. M. Brodsky, and R. G. W. Anderson. 1987. Assembly of clathrin-coated pits onto purified plasma membranes. *Science (Wash. DC).* 236:558–563.
Mostov, K. E., and N. E. Simister. 1985. Transcytosis. *Cell.* 43:389–390.
Octave, J. N., Y. J. Schneider, A. Trouet, and R. R. Crichton. 1983. Iron uptake and utilization by mammalian cells. I. Cellular uptake of transferrin and iron. *Trends Biochem. Sci.* 8:217–220.
Pearse, B., and M. S. Bretscher. 1981. Membrane recycling by coated vesicles. *Annu. Rev. Biochem.* 50:85–101.
Pearse, B. M. F. 1985. Assembly of the mannose-6-phosphate receptor into reconstituted clathrin coats. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:2457–2460.
Pearse, B. M. F., and R. A. Crowther. 1987. Structure and assembly of coated vesicles. *Annu. Rev. Biophys. Biophys. Chem.* 16:49–68.
Pearse, B. M. F. 1987. Clathrin and coated vesicles. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2507–2512.
Pypaert, M., J. M. Lucocq, and G. B. Warren. 1987. Coated pits in interphase and mitotic A431 cells. *Eur. J. Cell Biol.* 45:23–29.
Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 17:208–213.
Robinson, M. S., and B. M. F. Pearse. 1985. Immunofluorescent localization of 100K coated vesicle proteins. *J. Cell Biol.* 102:48–54.
Roth, J. 1983. The colloidal gold marker system for light and electron microscopic cytochemistry. *In* Techniques in Immunocytochemistry. Vol 2. G. R. Bullock and P. Petrusz, editors. Academic Press, Inc., London. 217–284.
Rothman, J. E., and S. L. Schmid. 1986. Enzymatic recycling of clathrin from coated vesicles. *Cell.* 46:5–9.
Siegel, S. 1956. The Mann-Whitney U test. *In* Nonparametric Statistics for the Behaviorial Sciences. McGraw-Hill Inc, New York. 116–127.
Sandvig, K., S. Olsnes, O. W. Petersen, and B. van Deurs. 1987. Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell Biol.* 105:679–689.
Sterio, D. C. 1984. The unbiased estimation of number and sizes of arbitrary particles using the disector. *J. Microsc.* 134:127–136.
Tycko, B., and F. R. Maxfield. 1982. Rapid acidification of endocytic vesicles containing α_1-macroglobulin. *Cell.* 28:643–651.
Warren, G. B., P. Woodman, M. Pypaert, and E. Smythe. 1988. Cell-free assays and the mechanism of receptor-mediated endocytosis. *Trends Biochem. Sci.* 13:462–465.
Woodman, P. G., and G. B. Warren. 1988. Fusion between vesicles from the pathway of receptor-mediated endocytosis in a cell-free system. *Eur. J. Biochem.* 173:101–108.