Exocytosis of Pinocytic Contents by Chinese Hamster Ovary Cells

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ABSTRACT The extent of exocytosis of pinocytic vesicle contents was studied in suspension-cultured Chinese hamster ovary (CHO) cells using horseradish peroxidase (HRP) as a pinocytic content marker. HRP was shown to be internalized via fluid-phase pinocytosis in CHO cells. After an HRP pulse of 2.5–10 min a rapid decrease of 30–50% in cell-associated HRP activity was observed within 10–20 min at 37°C. During this time the loss of cell-associated HRP was accompanied by an equivalent increase in extracellular HRP. After this rapid exocytosis of HRP, the remaining peroxidase activity decreased with a $t_{1/2}$ of 6–8 h, the known lysosomal half-life of HRP. In pulse-chase experiments HRP was chased into a nonexocytic compartment. Based on cell fractionation and electron microscopic experiments, this nonexocytic compartment was identified as a lysosome and the compartment from which exocytosis occurs as a pinosome. The occurrence of pinocytic content exocytosis in cultured fibroblasts suggests that exocytosis of pinocytic vesicle contents is a general phenomenon.

The fate of pinocytic vesicle contents is generally thought to be delivery to and ultimate degradation within a lysosomal compartment except in certain specialized cell types (capillary endothelium [2, 19], fetal yolk sac [11], and neonatal intestinal epithelial cells [14]). Based on time-lapse photomicrography studies, Gey et al. (4) speculated that pinocytized fluid whose fate could not be accounted for by transfer to other organelles was discharged from the cell. De Duve and Wattiaux (3), Gordon (5), and Morré et al. (10) have suggested that pinocytosis may be a bidirectional or multidirectional process and that rapid cycling, or exocytosis, of pinocytic contents to the extracellular medium may occur. Recent evidence has shown that endocytic membrane is rapidly cycled back to the cell surface in several cell types (12, 28, 30) and that internalized insulin is discharged from cultured fat cells into the extracellular medium (29). To date, no experimental test of the extent of exocytosis of pinocytic vesicle contents has been presented.

In this study we have directly measured the extent of exocytosis of pinocytic vesicle contents in Chinese hamster ovary (CHO) cells, a relatively nonspecialized cell type. Horseradish peroxidase (HRP) was chosen as the pinocytic marker because of its extremely high enzymatic activity which permitted the assay of nanogram quantities of enzyme in short-term uptake experiments. In addition, pulse-ingested HRP can be readily localized cytochemically and has no effect on cell viability. For these experiments, CHO cells were grown in suspension to permit the rapid removal of cells from exogenous reagents by centrifugation. The chief result of this work has been to demonstrate that between 30 and 50% of a pinocytic marker is exocytosed over a 10-min period in CHO cells.

MATERIALS AND METHODS

Cell Culture

Chinese hamster ovary (CHO) cells, an established hamster line of fibroblastic origin, were cultured in suspension in Eagle's Minimal Essential Medium, alpha modification (21) with 10% heat-inactivated fetal calf serum (αMEMFC10).

Quantification of Cell Number

Cell number was quantitated either with a hemacytometer or by use of a DNA-dependent, fluorescence enhancement assay (1).

HRP Internalization

Suspension-cultured CHO cells (2×10^6 cells/ml) in αMEMFC10 were exposed to 0.2–10 mg/ml HRP (type II, Sigma Chemical Co., St. Louis, Mo.) at 37° or 4°C. The internalization period was terminated by pouring the culture onto a volume of crushed, frozen NKMFC10 (0.13 M NaCl, 5 mM KCl, 1 mM $MgCl_2$ plus 10% heat-inactivated fetal calf serum) equal to one-fifth of the culture. The cultures were centrifuged at 200 *g* for 5 min at 2°C. The cells were then washed three times at 2–4°C in 10-ml volumes of NKMFC1 (NKM with 1% fetal calf serum), with a tube change each time. The cells were lysed with 0.05% Triton X-100 and frozen for later assay. HRP activity was assayed and expressed on a per cell basis.

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Effect of Sugars on HRP Internalization

Duplicate cultures of CHO cells (2×10^6 cells/ml) in αMEMFC10 supplemented with 50 U of penicillin and 50 μg streptomycin per ml (αMEMFC10-PS) were incubated at 37°C for 10 min in the absence or presence of the following saccharides: L-arabinose (10 mM), L-fucose (5 mM), D-galactose (25 mM), *N*-acetyl glucosamine (10 mM), D-mannose (25 mM), D-mannose-6-phosphate (10 mM), and yeast mannan (1 mg/ml). HRP was then added to a final concentration of 1 mg/ml. After a 10-min incubation at 37°C, HRP internalization was terminated as described above.

Assay for Exocytosis

After a brief HRP internalization period and three to four cold saline washes, the cells were resuspended in Puck's saline G (0.137 M NaCl, 5.36 mM KCl, 0.109 mM $CaCl_2$, 0.625 mM $MgSO_4$, 6.1 mM glucose, and 2.1 mM phosphate, pH 7.2), or saline G supplemented with 0.5 mg/ml bovine serum albumin. This is time t_0. The zero time point sample was resuspended at 4°C. All other samples were resuspended and incubated at 37°C. After various time intervals, the samples were centrifuged. The supernatant was removed and saved. The cells were lysed for 5 min with 0.05% Triton X-100. All samples were frozen and assayed later for enzyme activities and to determine cell number.

HRP Chase Conditions

CHO cells (2×10^6 cells/ml) in αMEMFC10 were exposed to 1 mg/ml HRP at 37°C for 10 min. After the internalization period, the culture was rapidly diluted 100-fold in 37°C αMEMFC10. This is the time t_0. After various time intervals, aliquots of cells were removed, poured onto one-twentieth volumes of crushed, frozen NKMFC10, and centrifuged at 4°C. The cells were washed three times at 4°C with NKMFC1 and lysed with 0.05% Triton X-100 for 5 min. Cell lysates were frozen and later assayed for HRP activity and cell number.

Enzyme Assays

HRP activity was assayed in 0.05% Triton X-100, 0.1 M phosphate buffer (pH 5.0) using *o*-dianisidine as a substrate (22). A Gilford Model 250 spectrophotometer with reference compensator was used. Lactate dehydrogenase activity was assayed at pH 7.3 by established procedures (Worthington Manual, 1977. Worthington Biochemical Corp., Freehold, NJ). Acid phosphatase activity was measured in 0.1% Triton X-100, 0.1 M acetate buffer (pH 5.0) using *p*-nitrophenyl phosphate as a substrate (6). β-hexosaminidase activity was measured in 0.1% Triton X-100, 0.1 M acetate buffer (pH 5.0) using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as a substrate (6).

Cytochemical Localization of HRP

CHO cells (4×10^6 cells/ml) in αMEMFC10 were incubated at 37°C with 1 mg/ml HRP for either 2.5, 10, or 120 min. CHO cells were also exposed to HRP for 2 h at 4°C. At the end of the internalization period, cells incubated at 37°C were poured onto crushed, frozen NKMFC10 equal to one-fifth the culture volume. Cells were then washed three times at 4°C with 10 ml of NKM with a tube change each time. The cells were fixed, washed, and incubated with diaminobenzidine (DAB) as a substrate for cytochemical localization of peroxidase activity as previously described (26). Samples were embedded in Spurr's resin and sectioned with a diamond knife. Sections were examined without lead citrate staining.

Cell Fractionation

CHO cells were incubated in αMEMFC10 at 37°C with 1 mg/ml HRP for 10 min. In some experiments after the internalization period, cells were chased for 2 h at 37°C as described above. Cells were washed three times with 4°C NKMFC1, once with 4°C Dulbecco's PBSA (0.137 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4), and three times with 4°C 0.25 M sucrose. Cells were disrupted by nitrogen cavitation (13, 15) as modified for suspension cultured CHO cells (Pool and Storrie, manuscript in preparation.) The resulting postnuclear supernatant was applied to a 30-ml 20–55% linear sucrose gradient over a cushion of 2.0 M sucrose and centrifuged in a Beckman SW27 rotor at 69,000 g_{av} for 2 h (Beckman Instruments, Inc., Fullerton, CA). These centrifugation conditions are similar to those described earlier by Steinman *et al.* (25). Fractions were collected by displacing the gradient with 2.5 M sucrose. Density values were determined by measuring the refractive index of fractions from a parallel gradient. All fractions were frozen and assayed later for HRP and β-hexosaminidase activity.

RESULTS

Mode of HRP Internalization

Experiments were conducted to determine HRP internalization conditions and to establish the mode of HRP uptake in CHO cells. If HRP uptake is by fluid-phase pinocytosis, the uptake should be directly proportional to time and to the concentration of HRP in the medium, insensitive to the presence of various sugars known to inhibit receptor-mediated pinocytosis, and detectable as a solute with no cell surface binding. These criteria were all met. First, HRP uptake was linear for 60 min and almost completely suppressed at low temperatures (Fig. 1). The low level of HRP seen in the 4°C sample did not increase with time. HRP ingestion was readily detected after internalization periods as short as 2.5 min. Second, during a 10-min uptake period HRP internalization was directly proportional to the concentration of exogenous HRP over the range of 0.2–10 mg/ml (Fig. 2). Third, incubation of cells with HRP in the presence of saccharides known to inhibit receptor-mediated pinocytosis (8, 16, 20) had no effect on the uptake of HRP, a glycoprotein (Table 1). Fourth, no cell surface binding of HRP was detected by electron microscopic cytochemistry after exposure of cells to peroxidase at either 4°C or 37°C (Fig. 3). These results validate the use of HRP as a pinocytic content marker in CHO cells.

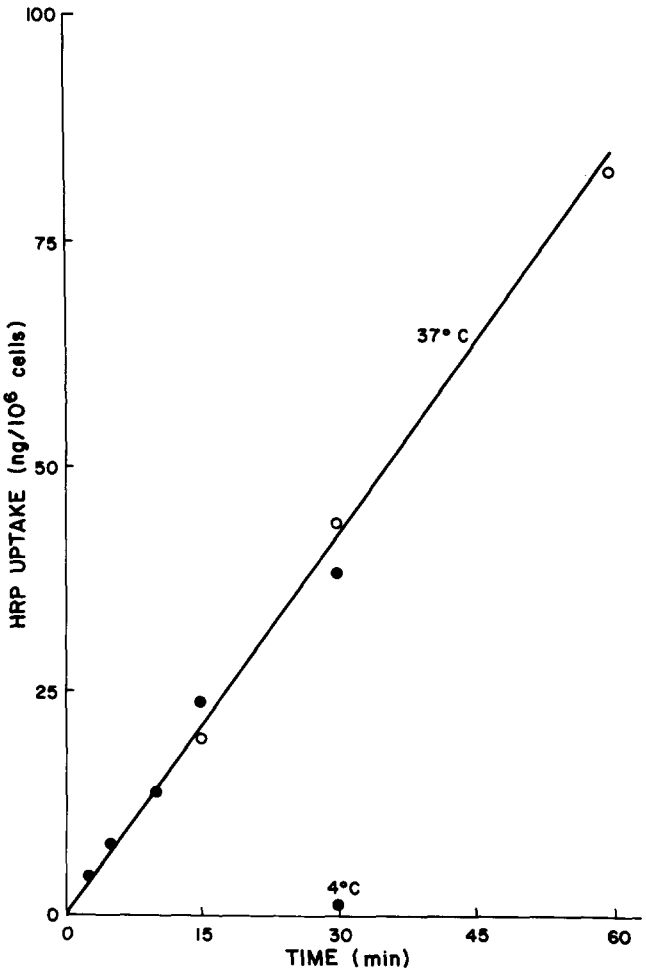

FIGURE 1 Kinetics of HRP uptake by CHO cells. Cells were exposed to 1 mg/ml HRP for various times, washed at 4°C, and assayed for cell-associated HRP. The data shown are from two separate experiments. The values plotted are the averages of duplicate samples.

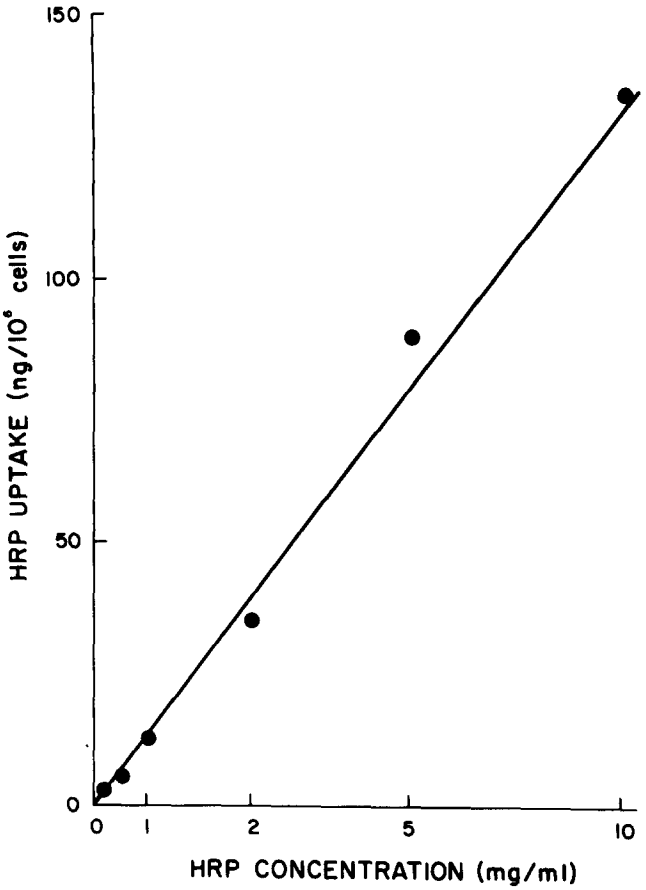

FIGURE 2 Dependence of HRP uptake by CHO cells on the concentration of exogenous HRP. Cells were incubated with various amounts of HRP for 10 min, washed at 4°C, and assayed for cell-associated HRP. The values shown are the averages of duplicate samples.

Fate of Pulse-ingested HRP

Two distinct alternatives for the fate of a pulse-ingested pinocytic content marker such as HRP are possible. Marker internalized into pinocytic vesicles could be quantitatively retained within the cell and transferred to secondary lysosomes through pinosome-lysosome fusion and, after a brief time, degraded with first-order kinetics as previously observed for proteins within secondary lysosomes (for review see references 9 and 17). Alternatively, exocytosis of a portion of pinocytic contents could occur, with the remainder being degraded in secondary lysosomes.

As a first step to distinguish between these two alternatives, CHO cells were exposed to HRP for 10 min at 37°C, washed at 4°C, incubated at 37°C, and assayed for cell-associated HRP at various times. Cells warmed in αMEMFC10 showed a biphasic curve for the loss of cell-associated HRP activity (Fig. 4). After 10 min, ~30% of the cell-associated HRP activity was lost. The remainder of the HRP activity was lost with an extrapolated half-life of 6 h. This value falls within the range of the lysosomal half-life of HRP (6–8 h) previously determined for CHO cells (27) and for other fibroblasts (24). In experiments in which the uptake period was shortened to 2.5, 5, or 7.5 min, no statistically significant difference in the relative extent of initial rapid loss of cell-associated HRP activity was observed.

The initial rapid loss of pulse-ingested HRP from CHO cells may be due to either rapid inactivation of HRP or to exocytosis of HRP. To distinguish between these two alternatives, cells were exposed to HRP for 10 min at 37°C, washed at 4°C, and warmed in 37°C saline G, a glucose-containing saline solution. Cell-associated and extracellular HRP activities were measured at various times. Again, the same biphasic kinetics for HRP loss from cells was observed (Fig. 5). The initial rapid loss was accompanied by a corresponding increase in extracellular HRP activity. Essentially all of the HRP activity was accounted for at all time points. Under these conditions no lactate dehydrogenase (LDH), β-hexosaminidase, nor acid phosphatase activity was observed in the extracellular medium. LDH is a soluble cytoplasmic enzyme which would be released into the medium

TABLE I

Effect of Saccharides on HRP Uptake

Addition	Uptake ng/10⁶ Cells	Uptake Relative
None	17.2	1.00
L-Arabinose (10 mM)	18.6	1.08
L-Fucose (5 mM)	18.4	1.07
D-Galactose (25 mM)	21.5	1.25
N-Acetyl glucosamine (10 mM)	16.0	0.93
D-Mannose (25 mM)	15.7	0.91
D-Mannose-6-phosphate (10 mM)	19.8	1.15
Yeast mannan (1 mg/ml)	14.3	0.83

Cells were preincubated with or without the above saccharides for 10 min in αMEMFC10-PS. HRP was added to a final concentration of 1 mg/ml. After 10 min, cells were then washed, lysed with Triton X-100, and assayed. The values reported are the averages of duplicate samples. The concentrations of saccharides used are the highest concentrations which have little or no effect on the growth of CHO cells.

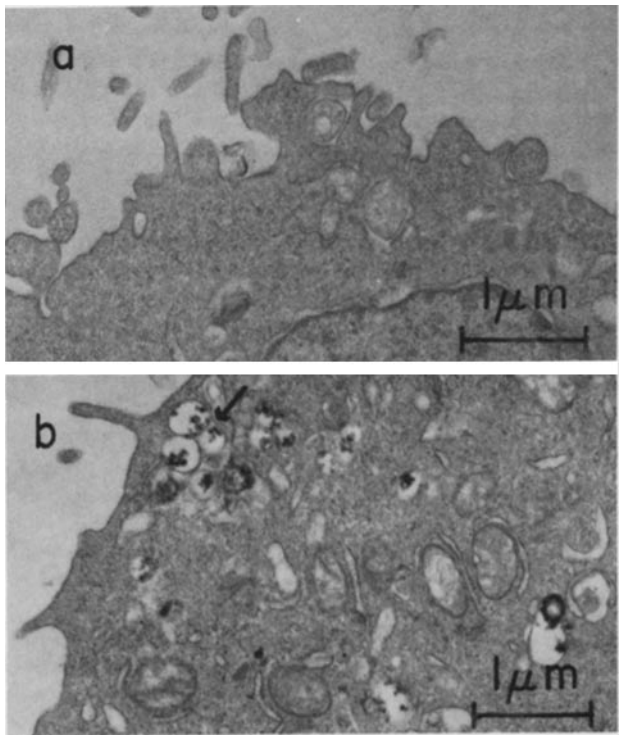

FIGURE 3 Lack of cell-surface binding of HRP on CHO cells. Cells were exposed to 1 mg/ml HRP for (*a*) 2 h at 4°C or (*b*) 2.5 min at 37°C. Arrow points to examples of cytochemical deposits localized in areas of HRP activity within vesicles. Sections were examined without lead staining. × 15,000.

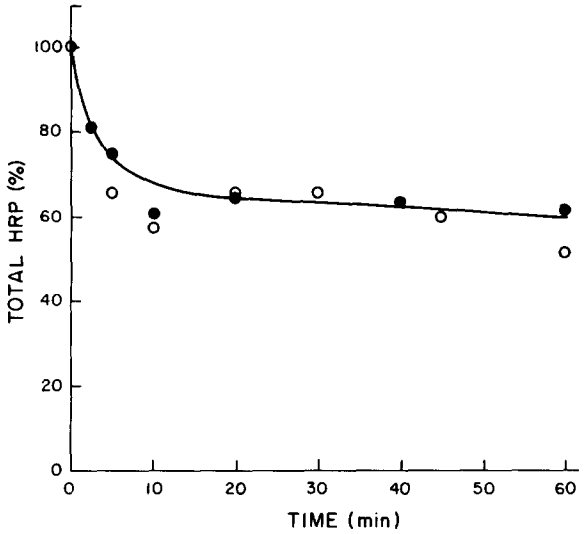

FIGURE 4 Fate of cell-associated HRP. Cells were incubated with 1 mg/ml HRP for 10 min, washed at 4°C, resuspended in αMEMFC10 at 37°C (t_0), and incubated for various times. The data shown are from two separate experiments. Total HRP activity at t_0 is normalized to 66.5 ng (13.3 ng/10^6 cells). The values of cell-associated HRP activity are the averages of duplicate samples.

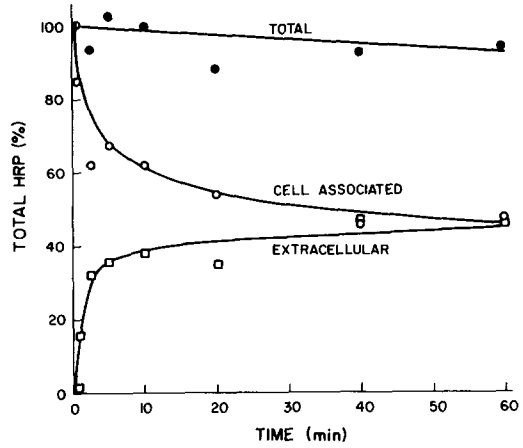

FIGURE 5 Exocytosis of ingested HRP. Cells were exposed to 1 mg/ml HRP for 10 min, washed at 4°C, resuspended in 37°C saline G (t_0), and incubated for various times. Cell-associated HRP activity (○) and extracellular (media) HRP activity (□) were measured. The sum of cell-associated HRP activity and extracellular HRP activity is shown (●). Values plotted are the averages of duplicate samples. Total HRP activity at t_0 is 65.4 ng (14.0 ng/10^6 cells). The variation between duplicates is less than or equal to the size of the symbols.

upon cell lysis. β-Hexosaminidase and acid phosphatase are lysosomal matrix enzymes (7). Therefore, under conditions in which content exocytosis occurs, lysosomal matrix enzymes are not exocytosed and cell breakage does not occur. The exocytosis of HRP observed in these experiments appeared to be a temperature-dependent process. When cells were resuspended and incubated in 4°C saline G, there was no change in cell-associated or extracellular HRP activity (data not shown).

To test whether the HRP exocytosis observed in these experiments was induced by the cell handling procedures, experiments assaying cellular HRP levels during physiological chase conditions were performed. Following the HRP ingestion period, a physiological chase was achieved by using a rapid, 100-fold dilution of the culture with 37°C αMEMFC10. The advantage of the dilution protocol is that the amount of a fluid-phase content marker ingested during a chase period is vastly reduced without cooling the cells at 4°C. Under these chase conditions the quantity of HRP ingested during a 30-min chase period amounts to only 3% of the quantity of HRP ingested during a 10-min pulse period. For cells exposed to HRP for 10 min and then diluted 100-fold to terminate the pulse period, the same biphasic loss of cell-associated HRP was observed (Fig. 6).

Subcellular Localization of HRP

HRP was detected within round, membrane-limited vesicles by electron microscopic cytochemistry after an ingestion period of 2.5 min (Fig. 3*b*). After a 10-min internalization period, the predominant class of HRP-positive vesicles was characterized by a peripheral rim of reaction product (Fig. 7*a*). To investigate the site of HRP accumulation in CHO cells, cells were exposed to HRP for long periods. After a 2-h internalization period most of the HRP-positive vesicles were filled with reaction product (Fig. 7*b*). Rimmed and filled vesicles have been previously described by Steinman et al. (23, 24) as pinosomes and secondary lysosomes, respectively. HRP was not detected at the cell surface.

Cell-fractionation experiments were done to determine the subcellular location of HRP both immediately after a 10-min uptake and after a 10-min uptake followed by a 2-h chase. Cells were homogenized by nitrogen cavitation, and total post-nuclear supernatants were fractionated in 20–55% linear sucrose gradients. The HRP and β-hexosaminidase activities found in the first few fractions of the gradients (Fig. 8) were an artifact caused by vesicle breakage during homogenization. After a 10-min uptake the bulk of the HRP activity peaked at a density of 1.115 (Fig. 8*a*). The HRP distribution observed after a 10-min uptake was clearly distinct from that of β-

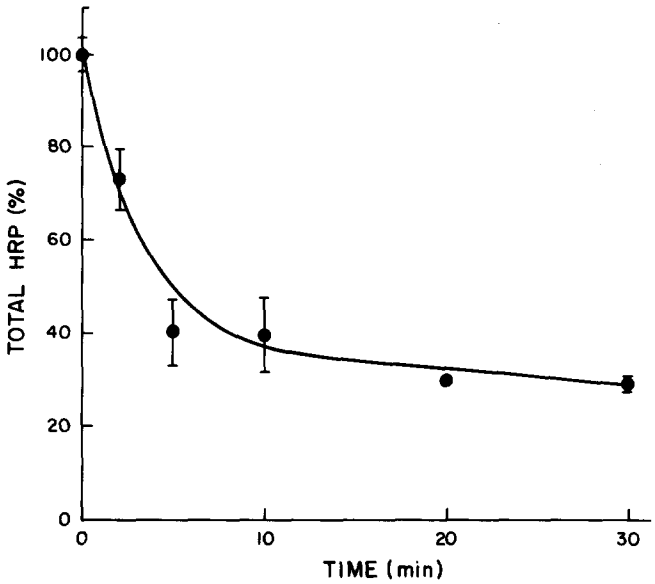

FIGURE 6 Fate of cell-associated HRP under physiological conditions. Cells were exposed to 1 mg/ml HRP for 10 min, rapidly diluted 100-fold with αMEMFC10 at 37°C (t_0), and chased for various times. The values for the cell-associated HRP activity (●) are the averages of duplicate samples. Total HRP activity at t_0 is 70.4 ng. Error bars are shown where the variation between duplicates is larger than the figure symbols.

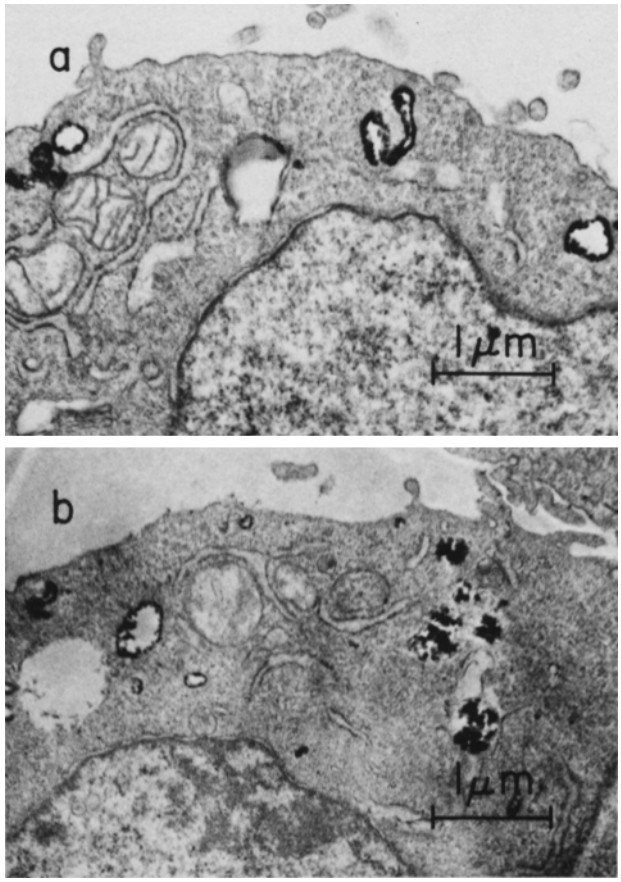

FIGURE 7 Cytochemical localization of HRP in CHO cells. Cells were exposed to 1 mg/ml HRP for (*a*) 10 min or (*b*) 2 h. The black deposits indicate sites of HRP activity. Sections were examined without lead staining. × 15,000.

hexosaminidase, a lysosomal marker.[1] After a 10-min HRP pulse followed by a 2-h chase, the distribution of HRP activity shifted from a density of 1.115 to 1.216 (Fig. 8*b*). After the 2-h chase the HRP activity corresponded closely to the β-hexosaminidase activity.

Steinman and Cohn (22) have reported that exocytosis does not occur from secondary lysosomes. To test whether this is true in CHO cells, experiments to assay for HRP exocytosis after a 2-h chase period were conducted. Cells were exposed to HRP for 10 min, diluted 100-fold with 37°C αMEMFC10, and incubated for 2 h. After the chase period, cells were assayed for exocytosis. No exocytosis of HRP occurred (data not shown).

DISCUSSION

This study was done to determine whether a fluid-phase pinocytic marker in CHO cells, a nonspecialized cell type, is quantitatively retained and transferred to a lysosomal compartment or whether some portion of the marker is exocytosed. Our results support two major conclusions. First, exocytosis does occur from a pinosomal compartment and is responsible for the rapid loss of >30% of the pulse-ingested content marker, HRP. Second, the remainder of the marker is transferred to secondary lysosomes from which exocytosis does not occur. This results in a biphasic curve for the loss of cell-associated HRP activity in which there is a rapid loss of >30% of the activity during the first 10 min, followed by a slow loss of activity. In these cells the initial rate of accumulation of HRP is equal to the rate of endocytosis minus the rate of exocytosis.

HRP, an easily assayed enzyme, was chosen as the pinocytic content marker. For CHO cells, HRP was shown to be internalized by fluid-phase pinocytosis with no detectable cell surface binding. These results are consistent with the observations of others (for review, see reference 17). Exocytosis of pulse-ingested content marker was essentially complete over a 10- to 20-min period. During this period, the appearance of extracellular HRP was sufficient to account for the decrease in cell-associated HRP. The extracellular appearance of the enzyme was not due to cell lysis. Previous investigators (22) have typically assayed for content exocytosis after a 30-min chase

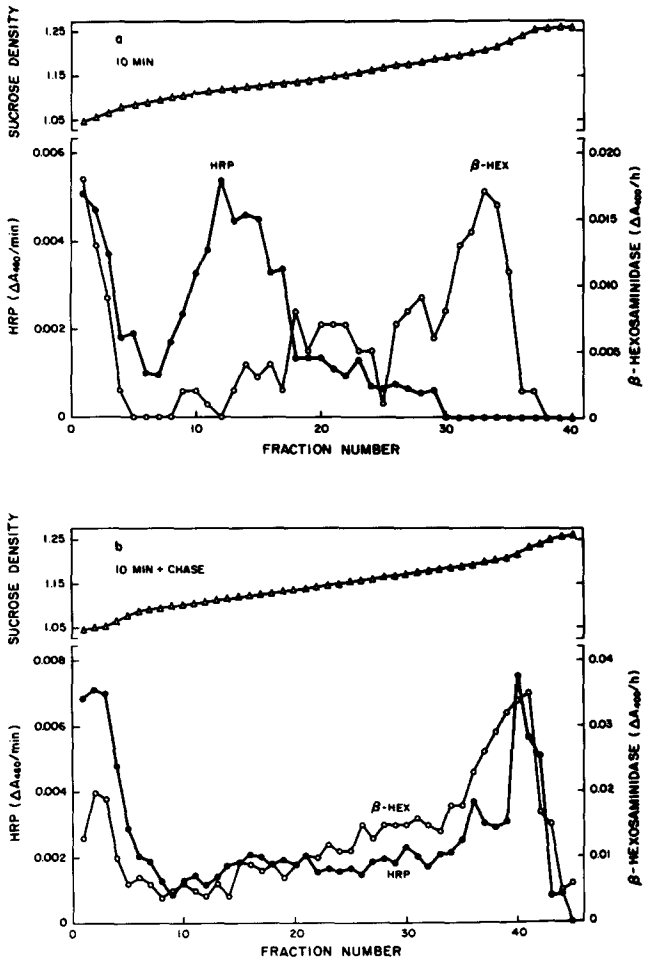

FIGURE 8 Distribution of HRP activity in sucrose gradients. CHO cells were exposed to 1 mg/ml HRP for (*a*) 10 min or (*b*) 10 min followed by a 2-h chase. Cells were homogenized by nitrogen cavitation, and total postnuclear supernatants were applied to 30-ml linear 20–55% sucrose gradients with 6.5-ml cushions of 2 M sucrose. Gradients were centrifuged in a Beckman SW27 rotor for 2 h at 69,800 g_{av} (Beckman Instruments, Inc.). Fractions were collected and analyzed for HRP (●) and β-hexosaminidase (○) activities. Density values (Δ) were determined by measuring the refractive index of fractions of a parallel gradient.

[1] Pulse-ingested HRP is associated with a distinct organelle population in CHO cells. By Percoll gradient centrifugation the HRP distribution observed after a 10-min uptake is clearly different from both plasma membrane and lysosomes (Pool, R. R., Jr. and B. Storrie, manuscript in preparation.).

period and consequently have failed to observe exocytosis. HRP exocytosis was temperature dependent as expected for a physiological process.

By both density gradient centrifugation and electron microscopy, the compartment from which exocytosis occurs was identified as a pinosome and the compartment in which HRP accumulates was identified as a lysosome. No exocytosis occurred from secondary lysosomes in agreement with the observations of Steinman and Cohn (22). The kinetics of the exocytic process indicate that transfer of contents into a nonexocytic compartment should be complete over a 10–20 min period. In preliminary experiments, we find that the distribution of pulse-ingested HRP in a sucrose density gradient after a 20-min chase period indicates the existence of density intermediates in the transfer of contents into lysosomes (Maurey, Pool, and Storrie, unpublished observations). Intermediates for receptor-mediated pinocytosis have been proposed by Willingham and Pastan (31).

The exocytosis of 30–50% of pulse-ingested pinocytic contents by CHO cells suggests that either a major fraction of the pinosome population never fuses with lysosomes or that for individual pinosomes the transfer of contents to lysosomes is an incomplete process with residual pinosome contents being exocytosed. Two models have been presented for the transfer of pinocytic vesicle contents to lysosomes. In the first (for review, see reference 17), fusion of pinosomes and lysosomes occurs with presumably quantitive transfer of vesicle contents. In the second (piranhalysis, reference 32), lysosomes repeatedly collide with pinosomes and gradually transfer pinocytic contents. Content exocytosis can be incorporated into either model for transfer of pinocytic contents into lysosomes. Pinocytic content exocytosis could occur from either pinosomes that have never collided with lysosomes or from pinosomes that retain residual contents.

The time period over which exocytosis occurs is similar to the time period over which endocytic membrane has been directly demonstrated to cycle to the cell surface in fibroblasts (28) and in other cells (12, 30). Exocytosis of pinocytic contents may occur as part of endocytic membrane cycling. In certain specialized cell types such as capillary endothelium (2, 19), fetal yolk sac (11), and neonatal intestinal epithelial cells (14), exocytosis of pinocytic contents has previously been described. In these cell types pinocytic content exocytosis constitutes a physiological transport process for the movement of macromolecules from one side of a tissue layer to another. This transport process has been termed transcytosis (18). The occurrence of pinocytic content exocytosis in a relatively nonspecialized cell type such as cultured fibroblasts suggests that exocytosis of pinocytic contents is a more generalized phenomenon than previously thought.

ADDENDUM

Using [^{14}C]sucrose, exocytosis of pinocytic vesicle contents has been demonstrated by Besterman et al. (*J. Cell Biol.* 91:716–727) for cultured fibroblasts and macrophages.

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