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UPTAKE, INTRACELLULAR TRANSPORT AND RELEASE OF ^{125}I-POLY(VINYLPYRROLIDONE) AND [^{14}C]-SUCROSE-ASIALOFETUIN IN RAT LIVER PARENCHYMAL CELLS

EFFECTS OF AMMONIA ON THE INTRACELLULAR TRANSPORT

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Abstract—We have studied the intracellular transport of ^{125}I-labeled poly(vinylpyrrolidone) (^{125}I-PVP) and [^{14}C]sucrose-asialofetuin (^{14}C-SAF) in isolated rat hepatocytes. ^{125}I-PVP and ^{14}C-SAF are taken up in the cells by fluid phase and receptor-mediated endocytosis, respectively. The labeled degradation products formed from ^{14}C-SAF are trapped in the lysosomes. They can therefore serve as markers for lysosomes in subcellular fractionation studies.

The accumulation of ^{125}I-PVP in the cells was rapid initially and then decreased to a constant value. The diminished rate of accumulation was due to release (exocytosis‡) of previously endocytosed ^{125}I-PVP. The release of ^{125}I-PVP was studied in cells that had accumulated ^{125}I-PVP for various times and then after washing incubated in new medium at 37°. About 25% of the radioactivity associated with the cells after 1 hr was released to the medium subsequently. No such release was observed in cells that had taken up ^{14}C-SAF.

Subcellular distribution of ^{125}I-PVP and ^{14}C-SAF was studied by isopycnic centrifugation in sucrose gradients. Both compounds were sequentially associated with light (1.13 g/ml) and dense (1.19 g/ml) vesicles. Exocytosed ^{125}I-PVP was derived from the light vesicles. The denser organelles were probably lysosomes as their distribution coincided with that of lysosomal enzymes. By measuring radioactivity soluble and precipitable in trichloroacetic acid it could be shown that only degraded ^{14}C-SAF was associated with lysosomes. Undegraded ^{14}C-SAF was associated with vesicles banding at 1.13 g/ml. Degraded ^{14}C-SAF was, however, also seen first in this region of the gradient, suggesting that degradation started in a light lysosome.

Both uptake and release of ^{125}I-PVP were temperature dependent; both processes ceased at 10°. Ammonium ions had negligible effects on uptake and release of ^{125}I-PVP. The amine inhibited, however, the transfer of both ^{125}I-PVP and ^{14}C-SAF to the lysosomes.

The liver plays a central role in controlling the blood level of many macromolecules: not only does the liver secrete many plasma-proteins but it also removes a wide variety of proteins (and other macromolecules) from the blood by receptor-mediated endocytosis. The two processes, secretion and endocytosis, necessitate a high rate of plasma-membrane recycling. Secretion carries membrane to the cell surface while endocytosis internalizes plasma-membrane. One way of getting insight into hepatic turnover of plasma membrane is to follow fluid phase endocytosis in the hepatic cells. During formation of the endocytic vesicle some extracellular fluid with its content of macromolecules is trapped. If the macromolecule does not adsorb to the membrane, the internalization of a given macromolecule would be expected to be directly proportional to its concentration in the cell environment. The fate of the endocytic vesicle (the endosome) varies from cell to cell. Eventually, some of its content may be brought to the lysosomes. However, in some cells some of the content of macromolecules is brought back to the plasma membrane and exocytosed to the medium [1, 2].

To use a molecule as a marker for fluid phase endocytosis some criteria should be fullfilled: the marker should be easy to quantitate, it should not adsorb to the membrane and its uptake should be directly proportional to its extracellular concentration. The marker should not be degraded intracellularly, so that its intracellular localization can be followed by subcellular fractionation techniques or microscopy. Many earlier reports showed that the uptake of fluid phase markers was strictly linear with time. However, recycling of marker to the medium may occur rapidly [1, 3], and could easily have been overlooked.

In the present report we have studied fluid phase endocytosis in isolated rat liver parenchymal cells. As a marker for the fluid phase of the forming endocytic vesicle we have used polyvinylpyrrolidone labeled with ^{125}I (^{125}I-PVP).§ It has been shown earlier that this molecule is taken up into cells with-

‡ Exocytosis is defined as release of contents from an intracellular vesicle after fusion between the plasma membrane and the membrane of the vesicle. Alternative terms are: retroendocytosis and reversed endocytosis.

§ Abbreviations used: ^{125}I-PVP, ^{125}I-poly(vinylpyrrolidone); ^{14}C-SAF, [^{14}C] sucrose-asialofetuin.

out being adsorbed to the membrane [4–6]. It is not degraded in lysosomes and will therefore accumulate in the cells during endocytosis. The present report confirms that ^{125}I-PVP is taken up into rat hepatocytes by fluid phase endocytosis. Following its uptake some of the marker is released from the cells by exocytosis. By subcellular fractionation in sucrose gradients it could be demonstrated that ^{125}I-PVP accumulates in lysosomes. Early after uptake the marker is in a buoyant organelle and part of the ^{125}I-PVP is released from this organelle to the medium.

We have also, in parallel experiments, studied the uptake and subcellular distribution of asialofetuin, labeled with ^{14}C-sucrose according to Pittman *et al.* [7]. The intracellular degradation of this ligand leads to degradation products that are trapped at the site of degradation (the lysosomes). Therefore, its subcellular distribution and possible release from the cells can be compared to that of ^{125}I-PVP. Our data indicate that the intracellular distributions of [^{14}C]-sucrose-asialofetuin (^{14}C-SAF) and ^{125}I-PVP are similar. However, the asialoglycoprotein is not released from the cells.

MATERIALS AND METHODS

Chemicals. Polyvinylpyrrolidone (PVP), fetuin, enzyme substrates, and collagenase were from Sigma. ^{125}I-polyvinylpyrrolidone (^{125}I-PVP), specific activity 25 μCi/ml (2.6 mg/ml), and [^{14}C]sucrose, specific activity 477 μCi/mole, were from Amersham Corp. [^{14}C]sucrose-asialofetuin (^{14}C-SAF) was prepared according to Pittman *et al.* [7], as described earlier [8].

Subcellular fractionation. Rat hepatocytes were fractionated by isopycnic centrifugation in sucrose gradients, as described earlier [9]. The cells were homogenized in a 0.25 M sucrose solution, and a postnuclear fraction was prepared by centrifuging the homogenate at 2000 *g* for 2 min. The nuclear fraction was washed once. Four ml of the combined supernatants (postnuclear fraction) were layered onto linear sucrose gradients (20–58% (w/w) sucrose) in 40 ml centrifuge tubes fitting the Beckman SW 27 rotor. The volume of the gradient itself was 34 ml. The gradients were centrifuged for 4 hr at 85,000 *g*. Following centrifugation, the gradients were divided into 20 fractions.

Cells. Isolated rat hepatocytes were prepared essentially according to Seglen [10] as described earlier [11].

Cell incubations. The isolated hepatocytes were suspended in a simple salt medium [12] containing 1% (w/v) bovine serum albumin. Cell concentration was 8–12 $\times$ 10^6 cells per ml. The volume of the cell suspension was usually 5 ml. Cell viability was always $\geq$ 90% as judged by the Trypan blue exclusion test. Incubations were carried out in 100 ml Erlenmeyer flasks, in a shaking water bath.

Measurement of cell-associated radioactivity. To assess uptake of [^{14}C] SAF the cells were separated from the medium by centrifugation through a layer of oil (dibutyl phthalate) in 750 μl tubes in a Beckman microfuge. The procedure has been described earlier [13]. This method is not suitable for measuring cellular uptake of a compound internalized by fluid endocytosis. The uptake by fluid endocytosis is extremely low in comparison with uptake mediated through the asialoglycoprotein receptor and centrifugation through oil traps too much medium between the cells in the pellet. To measure cell-associated ^{125}I-PVP 0.5 ml of the cell suspension (about 5 $\times$ 10^6 cells) was first diluted to 5 ml by adding incubation medium and centrifuged in 5 ml centrifuge tubes at 100 *g* for 1 min. The cell pellet was washed twice by centrifugation in 5 ml medium. Further centrifugations were found not to reduce cell-associated radioactivity. ^{125}I-PVP was found to bind to the plastic centrifuge tubes unless albumin (1%, w/v) or cold PVP (1 mg/ml) was included in the medium. To circumvent this problem a known fraction of the washed cells could be pipetted into a new tube before counting the radioactivity in a gamma counter.

Degradation of ^{14}C-SAF was measured as acid-soluble radioactivity following treatment of cell samples or gradient fractions with 10% (w/v) trichloroacetic acid.

Measurement of radioactivity. Radioactivity from ^{125}I was determined in a gamma counter. Prior to determination of beta rays from ^{14}C, cell pellets were suspended in 0.05% SDS and the precipitates after treatment with TCA were dissolved in 6 M NaOH. All samples were finally mixed with 4 ml of water and 5 ml of Insta-Gel (Packard Instrument Co., Downers Grove, IL 60515).

Enzyme assay. The lysosmal enzyme, β-acetylglucosaminidase, was measured according to Barrett [14].

RESULTS

Uptake of ^{125}I-PVP as a function of time, cell concentration and PVP concentration

The uptake of ^{125}I-PVP (expressed as μl/hr/10^6 cells) was measured in the presence of PVP concentrations between 10 nM and 10 μM and in cell suspensions with cell concentrations varied between 0.5 and 20 million cells per ml. The uptake of ^{125}I-PVP was found to be independent of both cell and PVP concentrations when cell-associated radioactivity was measured after 30 min, 1 hr, and 2 hr. These findings are in accordance with earlier reports [5], and the results are not shown here. The method used here makes it possible to assess cell-associated radioactivity very precisely, and it is possible to determine the uptake of ^{125}I-PVP as a function of time even during the first minutes of uptake. The data obtained are depicted in Fig. 1, and reveal that the apparent rate of uptake shows a rapid initial phase. After 15–30 min the rate of uptake levels off and after 30 min it is fairly constant for the remainder of the incubation period.

Part of the endocytosed ^{125}I-PVP is exocytosed

Two possible explanations for the rapid initial uptake can be envisaged. First, the endocytic rate may decrease as the cells are incubated at 37°. This was found not to be the case; the time course for uptake was the same when ^{125}I-PVP was added to the cell suspension 0, 15, 30, or 60 min after the start of the incubation at 37°. Second, the rapid initial phase could express the real endocytic uptake of ^{125}I-

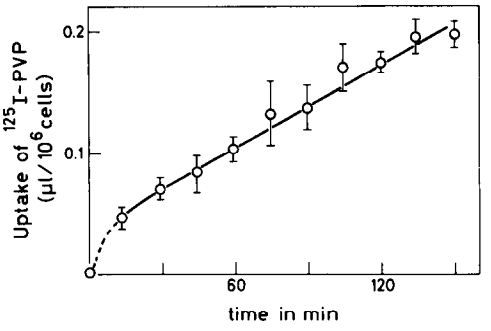

Fig. 1. Accumulation of ^{125}I-PVP in rat hepatocytes. The cells (10^7 cells/ml) were incubated at 37° in the presence of ^{125}I-PVP (1.25 µCi/ml/10 µM). Cell-associated radioactivity was measured at the indicated time-points and is presented as µl medium per 10^6 cells, i.e. the volume of medium containing the amount of ^{125}I-PVP that is associated with 10^6 cells at the given time point. The values presented are average ± S.E.M. for four determinations.

PVP, while the subsequent slower uptake is due to release (exocytosis) of previously endocytosed ^{125}I-PVP.

To test the latter hypothesis cells were incubated in the presence of ^{125}I-PVP at 37° for various times; they were then washed in ice-cold medium and thereafter incubated at 37° again. During the second incubation radioactivity associated with the cells and released to the medium was measured at various time points. To assess release to the medium resulting from cell rupture, β-acetylglucosaminidase and lactate dehydrogenase were measured together with cell viability. In parallel incubations cells were allowed to take up ^{14}C-SAF. The cells were after various times separated from the medium and reincubated in new medium (without the labeled ligand) at 37°. Again, as in the case of cells incubated with ^{125}I-PVP, radioactivity remaining in the cells and released to the medium was measured. The cells were treated with 5% (w/v) trichloroacetic acid, and acid soluble and acid precipitable radioactivities were measured.

The results of these experiments showed consistently that following the preincubation in the presence of ^{125}I-PVP the cells released a substantial amount of the radioactivity during the second incubation in the absence of labeled PVP in the medium. Figure 2 shows the results of an experiment in which the cells were preincubated for 0, 15, 30, and 60 min at 37°, before samples of cells were removed and release of labeled PVP followed. The data show that about the same amount of ^{125}I-PVP was released from cells preincubated 15, 30, or 60 min, and the half time for the release was in all cases about 5 min. After 60 min of preincubation with ^{125}I-PVP about 25% of the cell-associated radioactivity was subsequently released during 15 min. The released radioactivity corresponded to the decrease in cell-associated radioactivity. The release of ^{125}I-PVP was not due to cell rupture as no release of lysosomal enzymes or lactate dehydrogenase was detected (not shown). Cell viability also stayed unchanged during incubation.

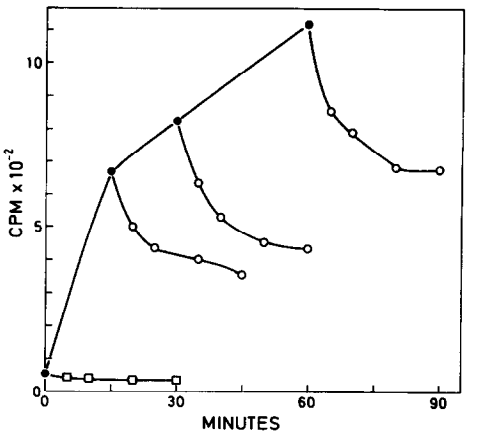

Fig. 2. Release of endocytosed ^{125}I-PVP from rat hepatocytes. Rat hepatocytes (10^7 cells/ml) were incubated at 37° in the presence of ^{125}I-PVP (1.25 µCi and 28 µg per ml). At the start of the incubation with ^{125}I-PVP, and after 15, 30 and 60 min cell aliquots were removed from the incubator and diluted with three volumes of ice-cold medium. The cells were sedimented and washed twice by centrifugation at 50 *g* for 30 sec and were then incubated again at 37° in isotope-free medium. Cell aliquots were taken at the start and 5, 10, 20 and 30 min after the start of the second incubation at 37°. The cells were centrifuged at 500 *g* for 1 min, and radioactivity released to the medium and remaining in the cells was determined. Cell-associated radioactivity in the release phase is indicated with open symbols (○, □).

Figure 3 shows that cells that had previously endocytosed ^{14}C-SAF for 30 min did not release this ligand to the medium subsequently. Therefore, the marker for fluid endocytosis is released back to the medium while that for receptor-mediated endocytosis is not. During a 30 min incubation of cells that had endocytosed ^{14}C-SAF in advance the acid soluble radioactivity in the cells increased from 20% to 50% of total cell-associated radioactivity (Fig. 3).

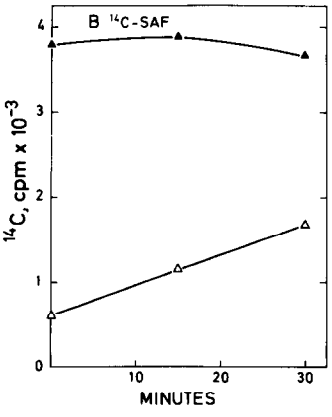

Fig. 3. Lack of release of endocytosed ^{14}C-SAF from rat hepatocytes. The cells were first incubated for 30 min with ^{14}C-SAF (100 nM) and then washed and reincubated in new medium without the labeled ligand. Cell-associated (▲) and acid soluble (△) radioactivity in the cells were measured after 0, 15 and 30 min.

This is a consequence of lysosomal degradation of the ligand and means that most of the ^{14}C-SAF taken up by the cells is directed to the lysosomes. The labeled degradation products formed do not penetrate the lysosomal membrane and are trapped in the cells.

Effect of temperature, rotenone and lysosomotropic amines on uptake and release of ^{125}I-PVP

Figure 4 shows that the uptake of ^{125}I-PVP, measured after 1 hr of incubation of the cells, decreased with decreasing temperatures; at 10° the uptake ceased completely. Release of endocytosed ^{125}I-PVP was also temperature-dependent. No release was demonstrated at 10° or below (Fig. 4).

Rotenone (100 μM) which inhibits electron transport reduced uptake of ^{125}I-PVP 50%. ATP concentrations in the cells were at the same time reduced from 10^{-7} to 5×10^{-10} M per 10^6 cells.

Lysosomotropic amines (chloroquine (100 μM) and ammonia (10 mM), and monensin (50 μM)), had only modest effects on the uptake and release of ^{125}I-PVP by the hepatocytes (data not shown).

Subcellular fractionation of cells endocytosing or exocytosing ^{125}I-PVP and ^{14}C-SAF

Cells were incubated with ^{125}I-PVP and ^{14}C-SAF at 37° for 1 hr and were then separated from the medium, washed in ice-cold medium and reincubated at 37° in the absence of extracellular ^{125}I-PVP or ^{14}C-SAF. Samples were taken from cells accumulating ^{125}I-PVP or ^{14}C-SAF, and from cells that were reincubated in absence of ^{125}I-PVP and ^{14}C-SAF. The cell samples were homogenized, and postnuclear fractions were prepared and centrifuged in linear sucrose gradients as described in the Materials and Methods section. In the fractions were measured radioactivity (in the case of fractions containing material from cells that had taken up ^{14}C-SAF both acid soluble and acid precipitable radioactivities were measured), β-acetylglucosaminidase, and densities (by way of refractive index). The results are shown in Fig. 5. The density distribution of radioactivity in gradients after centrifuging postnuclear fractions from cells taking up ^{125}I-PVP shows two distinct peaks (Fig. 5A), one at a density of 1.12–1.13 g/ml, and another at 1.19 g/ml. The latter peak is coinciding with that of the lysosomal enzyme (Fig. 5C) and presumably represents ^{125}I-PVP accumulating in lysosomes. The results obtained on cells that were incubated in the absence of extracellular ^{125}I-PVP after the one hour incubation in presence of labeled PVP showed a rapid decrease in radioactivity associated with the more buoyant organelle (Fig. 5B). At the same time radioactivity associated with lysosomes showed a slight increase. Most likely the two peaks in the gradients represent endosomes (density 1.12–1.13) and lysosomes (density 1.19), and when the cells are reincubated in medium without labeled PVP, radioactivity associated with endosomes is delivered partly to the lysosomes and partly to the medium (by exocytosis). Taken in conjunction with the results in Fig. 2, it is clear that ^{125}I-PVP is not released to the medium from the lysosomes.

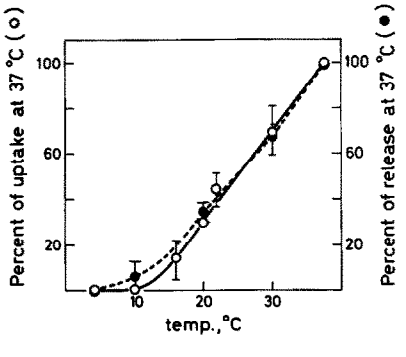

Fig. 4. Effect of temperature on the uptake (○) and release (●) of ^{125}I-PVP in rat hepatocytes. The uptake and release values are presented as per cent of values obtained at 37° in each experiment. Values ± S.E.M. for three experiments are given. The cells (10^7 cells/ml) were at each temperature incubated for 1 hr in presence (uptake) or absence (release) of ^{125}I-PVP (1.25 μCi/ml, 2.8 μg/ml).

The distribution of acid-soluble and acid-precipitable radioactivity in density gradients after centrifuging postnuclear fractions from cells endocytosing ^{14}C-SAF is shown in Fig. 5D–F. During the 60 min uptake period (Fig. 5D and E), undegraded (acid precipitable) ^{14}C-SAF accumulated in an organelle with a density of about 1.13 g/ml. The distribution of acid precipitable radioactivity was similar to the buoyant vesicle containing ^{125}I-PVP and presumably represents endosomes. Acid-soluble radioactivity also appeared first in the same region of the gradient as acid-precipitable radioactivity, as if degradation of the ligand was initiated in the endosomes (Fig. 5D). However, the labeled degradation products from ^{14}C-SAF later appeared in the gradient at the same position as β-acetylglucosaminidase (Fig. 5E). Negligible amounts of acid precipitable radioactivity were at any time found in this region of the gradient. Fractionation of cells that were first incubated with ^{14}C-SAF and then reincubated in the absence of the labeled ligand (Fig. 5F) showed that acid-soluble radioactivity associated with lysosomes increased, at the expense of acid precipitable radioactivity in the endosomes (density of 1.13). The radioactivity removed from the endosomes could be accounted for by the uptake in the lysosomes. No radioactivity was lost to the medium.

Ammonia inhibits the transfer of ^{125}I-PVP or ^{14}C-SAF from endosomes to lysosomes

It has been shown that ammonia, chloroquine, or monensin inhibits the transfer of asialoglycoproteins from endosomes to lysosomes [15–17] and thereby retard degradation of the ligand indirectly. The reason for this effect of the drugs is not understood. The fusion/contact between endosomes and lysosomes could be inhibited, or the drugs by reducing ligand–receptor dissociation may prevent the ligand from following the endosome to the lysosome. ^{125}I-PVP which does not adsorb to membranes should, on the other hand, be free to follow the endosomes during their intracellular transport. ^{125}I-PVP may therefore in subcellular fractionation studies serve to indicate transfer of endosomal contents to the

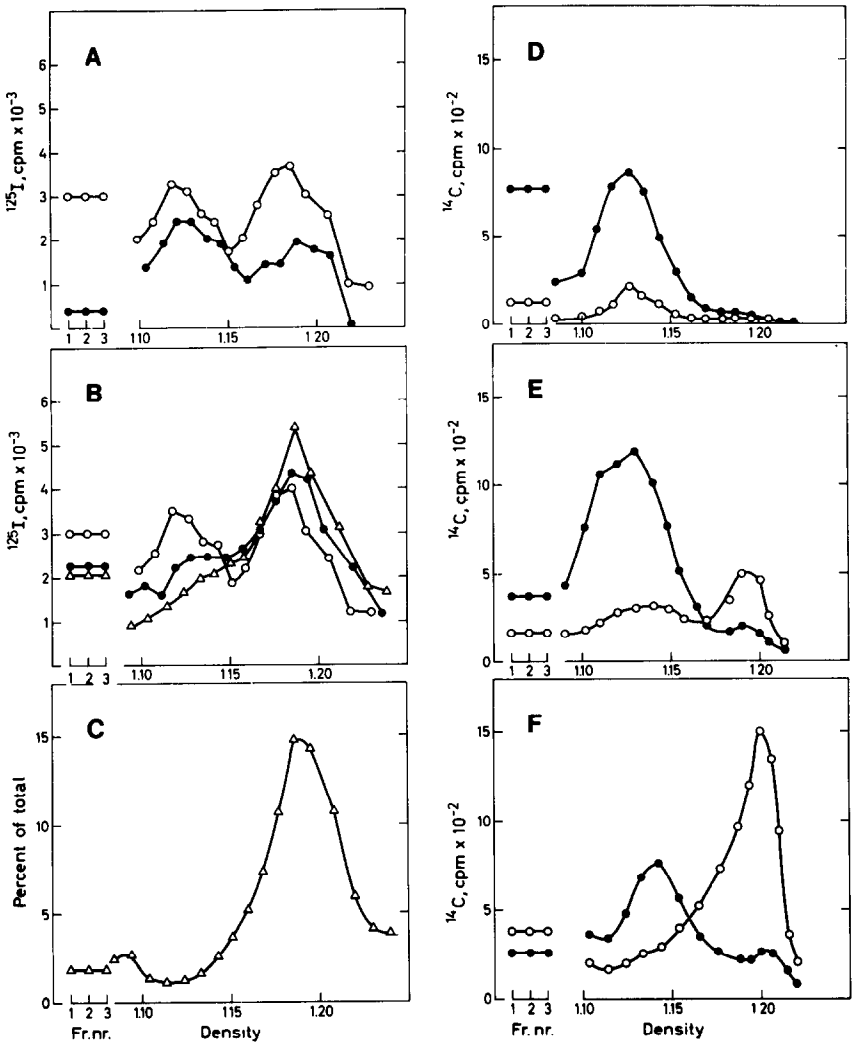

Fig. 5. Distribution of ^{125}I-PVP (A and B) and ^{14}C-SAF (D–F) in sucrose gradients. Cells were incubated with ^{125}I-PVP or with ^{14}C-SAF for 1 hr and were then separated from the medium and reincubated in isotope-free medium. Cell aliquots were removed during and after the uptake phase, homogenized, and fractionated by isopycnic centrifugation in linear, sucrose gradients. Subcellular distributions of ^{125}I-PVP are shown in panels A and B. In A are shown distribution curves for ^{125}I-PVP for cells incubated in presence of ^{125}I-PVP for 30 min (●) and 1 hr (○). Panel B shows distribution curves of ^{125}I-PVP after fractionating cells that were first incubated at 37° for 1 hr with ^{125}I-PVP (uptake phase) and then, after washing, incubated for 0 min (○), 15 min (●) and 30 min (△). Panel C shows the density distribution of β-acetylglucosaminidase after fractionating cells incubated for 1 hr in presence of ^{125}I-PVP (open circles in A or B). Panels D–F show distribution curves for acid-soluble (○) and acid precipitable (●) radioactivity after fractionating cells that were incubated with ^{14}C-SAF for 30 min (D) and 60 min (E), and then, after washing, incubated for 30 min in the absence of extracellular ligand (F).

lysomes. In the experiment depicted in Fig. 6 (A and D) cells took up ^{125}I-PVP for 1 hr in the presence (Fig. 6A) and absence (Fig. 6B) of 10 mM NH_4^+. The cells were then fractionated by sucrose gradient centrifugation and the isopycnic distribution of ^{125}I-PVP together with a lysosomal marker enzyme determined.

The distribution curves in Fig. 6B suggest—in accordance with the results in Fig. 5—that ^{125}I-PVP in control cells may be associated with endosomes and lysosomes, banding in the gradient at 1.13 and 1.20 g/ml, respectively. The peak of ^{125}I-PVP at the higher density coincides neatly with that of the lysosomal marker enzyme. In the ammonia-treated cells, on the other hand, very little radioactivity seemed to be associated with the lysosomes; most of the radioactivity was banding in the gradient at a density of about 1.13 g/ml. These data therefore indicate that ammonia inhibits the transfer of ^{125}I-PVP from endosomes to lysosomes.

For comparison we also studied the intracellular distribution of ^{14}C-SAF following treatment with NH_4^+. Figures 6C and D show the isopycnic distribution of acid soluble and acid precipitable ^{14}C-radioactivity in sucrose gradients after fractionating cells that took up ^{14}C-SAF for 60 min at 37° in

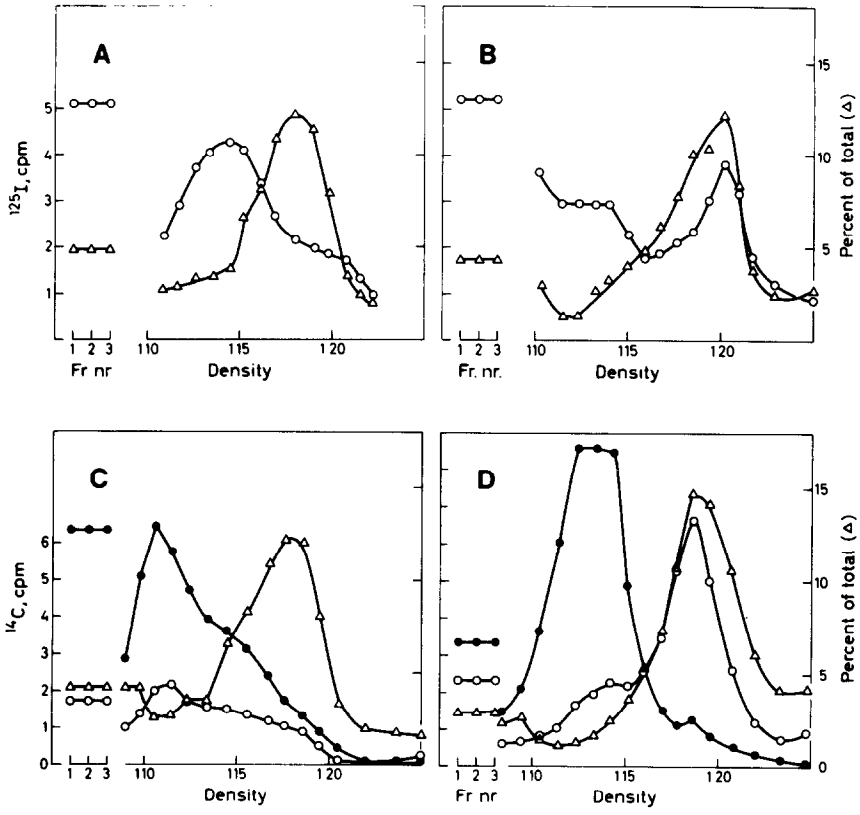

Fig. 6. Effects of ammonia on the subcellular distributions of ^{125}I-PVP, ^{14}C-SAF, and β-acetylglucosaminidase. Rat hepatocytes were incubated at 37° with either ^{125}I-PVP (A and B) or ^{14}C-SAF (C and D), and in the presence (A and C) or absence (B and D) of 10 mM NH_4^+. Panels A and B show the isopycnic distributions of ^{125}I-PVP (○) as c.p.m. (× 10^{-2}) per fraction while β-acetylglucosaminidase (△) in each fraction is presented as % of total recovered activity in the gradient. In panels C and D are shown acid soluble (○) and acid precipitable (●) radioactivity together with β-acetylglucosaminidase (△) after fractionating cells that had been incubated with ^{14}C-SAF in presence of absence of NH_4^+ (10 mM). Radioactivity is presented as c.p.m. (× 10^{-2}) per fraction while the lysosomal enzyme activity in each fraction is presented as per cent of total recovered activity in the gradient.

presence (Fig. 6C) or absence (Fig. 6D) of 10 mM NH_4^+. Ammonium ions not only led to changes in the density distribution of radioactivity, it also reduced the uptake of ^{14}C-SAF in the cells. Consequently, the amounts of both acid soluble and acid precipitable radioactivities recovered in the gradients were lower for the NH_4^+-treated cells than for the controls. In addition, NH_4^+ clearly inhibited degradation of ^{14}C-SAF and the relative amount of acid soluble radioactivity was much lower in the treated cells than in the control cells. The acid precipitable radioactivity was well separated from the lysosomal enzyme in the gradients after centrifuging postnuclear fractions from both NH_4-treated and control cells. Evidently, NH_4^+-treatment did not lead to accumulation of undegraded ligand in the lysosomes. Rather, NH_4^+ somehow interfered with the entrance of ligand into the lysosomes. This is in agreement with its effect on the intracellular distribution of ^{125}I-PVP. In control cells, on the other hand, ligand gained access to the lysosomes and the distribution curve for acid soluble radioactivity coincided quite well with that of the lysosomal marker enzyme, β-acetylglucosaminadase (Fig. 6D).

DISCUSSION

The present data show that a large fraction of ^{125}I-PVP endocytosed in isolated rat hepatocytes is subsequently exocytosed. A rapid initial uptake of labeled PVP corresponds to the time lag before exocytosis starts. After 15–30 min the rate of release of ^{125}I-PVP is constant and the apparent uptake in the cells is linear with time. The results of the cell fractionation experiments showed that ^{125}I-PVP was distributed between two types of organelles, one of these was probably lysosomes while the more buoyant vesicle(s) probably were endosomes in various stages of development. The organelle carrying ^{125}I-PVP back to the plasma membrane evidently was one such buoyant vesicle: when cells were incubated with ^{125}I-PVP and then separated from the medium and reincubated without ^{125}I-PVP, radioactivity was lost rapidly from the buoyant vesicles and at the same time appeared in the medium. The exocytosed ^{125}I-PVP is therefore not recycled through the lysosomes. Exocytosis of pinocytic contents has been demonstrated in fibroblasts and macrophages [1], and in Chinese hamster ovary cells [3]. In an earlier

report it was considered likely that part of the endocytosed ^{125}I-PVP in rat hepatocytes could be exocytosed [5]. However, the measurement of cell-associated ^{125}I-PVP in this study was not sufficiently precise to settle the point.

Both endocytosis and exocytosis were temperature-dependent as expected for physiological processes. The temperature effects as well as the effect of an inhibitor of energy metabolism are in accordance with earlier data [5]. Lysosomotropic amines as well as monensin had surprisingly small effects on the uptake and the release of ^{125}I-PVP in the hepatocytes. The lack of effect of monensin is in agreement with a recent report by Thilo and Burgert [18] who demonstrated that plasma membrane recycling in a macrophage cell line was unaffected by this ionophore.

Although ammonia did not affect the uptake of ^{125}I-PVP it influenced the intracellular distribution of ^{125}I-PVP. In its presence the transfer of labeled PVP from endosomes to lysosomes was inhibited. A similar effect of amines has been shown for the intracellular transport of compounds taken up by receptor-mediated endocytosis [15–17]. Tolleshaug and Berg proposed already in 1977 that ammonia and chloroquine inhibited intracellular degradation of ^{125}I-asialofetuin in rat hepatocytes by inhibiting the transfer of ligand from endosomes to lysosomes, and not by inhibiting the intralysosomal degradation as such [19]. This group found the same to be true for colchicine [20] and for monensin as well [17]. These conclusions were mainly based on cell-fractionation studies. The mechanism of this inhibition is not clear. In the presence of amines, pH in endosomes is elevated and receptor–ligand dissociation does not take place. Therefore, the ligand may follow the receptor when the receptors are segregated from the rest of the endosome and brought back to the cell surface. The remaining endosome may conceivably be brought to the lysosome. Another possibility is that ammonia by raising intra-endosomal pH and intralysosomal pH interferes with the fusion between endosomes and lysosomes or the transfer of endosomal contents to the lysosomes. If fluid endocytosis is a by-product of receptor-mediated endocytosis, then ^{125}I-PVP should follow the endosomes to the lysosomes, even if ligand went back with the receptor. The present data show that NH_4^+ inhibits transfer of ^{125}I-PVP from endosomes to lysosomes. Therefore, the amine seems to inhibit the interaction/fusion between endosomes and lysosomes.

The net uptake of fluid by fluid endocytosis of ^{125}I-PVP was 0.1 μl/hr per 10^6 cells which is in excellent agreement with earlier reports [5, 6].

The uptake of macromolecules by fluid endocytosis is very small in comparison with receptor-mediated uptake. Thus, 10^6 cells, suspended in 1 ml, may take up 0.01% of the fluid (and 0.01% of fluid markers in the medium) during 1 hr by fluid endocytosis. In comparison, in the presence of 20 nM asialoorosomucoid all the ligand is endocytosed during 1 hr. At this concentration of ligand receptor-mediated uptake is 10^5 times more efficient than fluid uptake. But even though the fluid uptake is modest it reflects a considerable amount of membrane internalization. If the initial vesicles formed are 0.2 μm in diameter 0.1 μl fluid/hr and 10^6 cells reflects an internalization of membrane corresponding to the whole cell surface per 30 min.

We do not know if ^{125}I-PVP is internalized into the same endosomes as ligands taken up by receptor-mediated endocytosis. There are, however, striking similarities between the two kinds of uptake: part of the endocytosed ^{125}I-PVP is exocytosed, indicating return of membrane to the cell surface. Similarly, after uptake of asialoglycoproteins, the receptors are returned tc the cell surface probably as part of a membrane vesicle.

At present, two pathways for returning asialoglycoprotein receptors to the cell surface seem to exist. First, the unoccupied receptor is returned after dissociation of ligand in the endosome (the "CURL" [21]). Second, a large fraction of endocytosed ligand–receptor complexes may be returned to the cell surface where the ligand may be released by adding EGTA [22, 23]. The latter observation suggests that part of the receptor–ligand complexes has not been recycled through the acidic endosome. Possibly, both return pathways may be available for pinocytosed ^{125}I-PVP.

However, the relationship between exocytosis of ^{125}I-PVP and receptor-recycling is not a simple one. Monensin has no effect on the release of ^{125}I-PVP but interferes with the return of the asialoglycoprotein receptors back to the cell surface [17]. Hence, the receptors are trapped intracellularly and the receptor number on the cell surface is reduced 50–70% [17]. Conceivably, the receptor pool affected by monensin may normally be routed through the Golgi while ^{125}I-PVP does not pass through this organelle. Monensin in low concentrations inhibit transport of secretory products through the Golgi [24]. One possibility is that ^{125}I-PVP is contained in the same vesicle as the ligand which is returned to the cell surface still bound to the receptor (the "slowly dissociating pool" [23]. The release of ligand after adding EGTA [23] and the exocytosis of ^{125}I-PVP have very similar kinetics. Interestingly, the EGTA-induced release of ligand is also unaffected by monensin (25 μM) or ammonia (10 mM) (Kindberg, unpublished data). These observations are in good accordance with a recent report by Mellman *et al.* [25]. They found that *monovalent* Fab—Fc receptor complexes in macrophages are internalized, delivered to endosomes and returned to the cell surface. This recycling was unaffected by ammonia and monensin.

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