Vanadate is a phosphate analogue that inhibits enzymes involved in phosphate release and transfer reactions (Simons, T. J. B. (1979) Nature 281, 337-338). Since such reactions may play important roles in endocytosis, we studied the effects of vanadate on various steps in receptor-mediated endocytosis of asialoorosomucoid labeled with ¹²⁵I-tyramine-cellobiose (¹²⁵I-TC-AOM). The labeled degradation products formed from ¹²⁵I-TC-AOM are trapped in the lysosomes and may therefore serve as lysosomal markers in subcellular fractionation studies. Vanadate reduced the amount of active surface asialoglycoprotein receptors ~70%, but had no effect on the rate of internalization and retroendocytosis of ligand. The amount of surface asialoglycoprotein receptors can be reduced by lowering the incubation temperature gradually from 37 to 15 °C (Weigel, P. II., and Oka, J. A. (1983) J. Biol. Chem. 258, 5089-5094); vanadate affected only the temperature-sensitive receptors. Vanadate inhibited degradation of ¹²⁵I-TC-AOM 70-80%. Degradation was much more sensitive to vanadate than binding; half-maximal effects were seen at -1 mM vanadate for binding and -0.1 mM vanadate for degradation. By subcellular fractionation in sucrose and Nycodenz gradients, it was shown that vanadate completely prevented the transfer of ¹²⁵I-TC-AOM from endosomes to lysosomes. Therefore, the inhibition of degradation by vanadate was indirect; in the presence of vanadate, ligand did not gain access to the lysosomes. The limited degradation in the presence of vanadate took place in a prelysosomal compartment. Vanadate did not affect cell viability and ATP content.

Vanadate has been shown to have a number of biological effects. Many of these effects are probably due to the fact that vanadate is a phosphate analogue which inhibits enzymes involved in phosphate transfer and release reactions. Enzymes inhibited by vanadate drive ATP-dependent ion pumps including Na⁺-K⁺-ATPase (Macara, 1986; Simons, 1979), Ca²⁺-Mg²⁺-ATPase (Delfert and McDonald, 1985; Yamanishi et al., 1984), and proton ATPase (Xie et al., 1989). It stimulates tyrosine phosphorylation of the β-subunit of the insulin receptor in adipocytes by inhibiting protein-phosphotyrosine phosphatases and/or by directly activating insulin receptor β-subunit autophosphorylation (Bemier et al., 1988; Kadota et al., 1987; Tamura et al., 1984). Vanadate has also been shown to inhibit dynein ATPase (Gibbons et al., 1988) and may therefore interfere with ciliary movements. A similar mechanism may be responsible for its effect on intracellular distribution and function of 10-nm filaments (Wang and Choppin, 1981).

Several steps in the endocytic process may depend on phosphate transfer and release reactions, and vanadate could therefore conceivably be used to obtain information about such steps. For instance, endocytic receptors may be phosphorylated on serine/threonine or tyrosine residues in their cytoplasmic tail, and the distribution of receptors between plasma membrane and an intracellular location may depend on the phosphorylation state of the receptor (Sibley et al., 1987). Increased internalization of epidermal growth factor (Berguino et al., 1985; Fearn and King, 1985), transferrin (Klausner et al., 1984; May et al., 1984), and asialoglycoprotein (Fallon and Schwartz, 1986, 1988) receptors has been observed after treatment of cells with phorbol esters which activate protein kinase C. Such data suggest a model in which receptors are phosphorylated on the cell surface, leading to internalization into a microsomal compartment where dephosphorylation can occur. Phosphorylation may conceivably occur in coated pits. Covera et al. (1988a) found that a highly serine/threonine-phosphorylated form of the IGF-II receptor resides in a membrane subfraction that contains virtually all of the clathrin associated with adipocyte plasma membrane. Clathrin-coated structures contain several protein kinase activity (Bar-Zvi and Branton, 1986; Keen et al., 1987; Usami et al., 1984), including casein kinase II (Bar-Zvi and Branton, 1986), which has been shown to phosphorylate the purified IGF-II receptor (Veron et al., 1988b).

Phosphorylation of the insulin receptor, stimulated by insulin or vanadate, may lead to receptor internalization (Backer et al., 1988; Hachiya et al., 1987; Marshall and Monzon, 1987; Torossian et al., 1988), and it has been suggested that vanadate, by stimulating phosphorylation of the β-subunit of the insulin receptor, diverts a proportion of insulin-receptor complexes from a retroendocytic to a degradative pathway (Marshall et al., 1987). Vanadate has been shown to increase the number of IGF-II receptors on the surface of adipocytes in a manner similar to insulin (Kadota et al., 1987). This effect of vanadate is probably due to its ability to activate the tyrosine kinase activity of the insulin receptor (Kadota et al., 1987). The redistribution of IGF-II from a low density microsomal compartment to the plasma membrane is, on the other hand, associated with a specific and dramatic decrease in phosphorylation state of the IGF-II receptors (Corvera and Cobb, 1986; Corvera et al., 1988a). The mechanism whereby insulin prevents phosphorylation is not clear. It could activate...
Vanadate Inhibits Endocytosis

a receptor phosphate or inhibit a kinase acting on the IGF-II receptor. Alternatively, insulin could exclude the receptor from regions of the membrane that contain the kinases (Corry et al., 1988a).

The intracellular transport of endocytic vesicles is dependent on an intact cytoskeleton and is inhibited by colchicine (Kolset et al., 1979; Oka and Weigel, 1983; Wolkoff et al., 1984). It has been shown that vanadate (0.1–1 μM) inhibits the Mg²⁺-ATPase activity of a microtubule-associated protein, dynein (Gibbons et al., 1988), thought to be the side-arm structure responsible for the sliding motile action of microtubules. Wang and Choppin (1991) studied the effect of vanadate on the organization of cytoskeletal structures in BHK21-F cells and found that it altered the distribution of 10-nm filaments and separated them from microtubules. Accompanying this change in cytoskeletal elements was an alteration in the distribution of organelles including lysosomes. These results indicated that vanadate separated 10-nm filaments and microtubules topologically and functionally and support the notion that 10-nm filaments and microtubules act together in the movement of cell organelles.

Vanadate may also affect intralysosomal degradation of proteins. Seglen and Gordon (1981) found that 10 mM vanadate strongly inhibits endogenous protein degradation as well as degradation of an exogenous endocytosed protein in isolated rat hepatocytes. It was found that the release of [¹⁴C]valine from radioactive protein labeled in vivo for 24 h is reduced ~70%. The fact that vanadate also inhibits degradation of long-lived proteins in homogenate from animals whose protein was labeled with [¹⁴C]valine for 24 h in vivo suggests that the inhibition by vanadate of lysosomal proteolysis is exerted on lysosomal cathepsins. A later report suggested that vanadate inhibits the action of cathepsin D, but that this is not due to a direct interaction with the enzyme. Rather, vanadate appears to interact with the protein substrates at acidic pH (Pillai and Zull, 1985).

The purpose of this study was to determine the effects of vanadate on the various steps of receptor-mediated endocytosis of asialoorosomucoid in rat liver parenchymal cells. The effects of vanadate on binding, internalization, retroendocytosis, continuous uptake, and degradation of ¹²⁵I-tyramine-cellobiose-labeled asialoorosomucoid (¹²⁵I-TC-AOM) were studied in isolated suspended rat liver parenchymal cells. The effects of vanadate on the intracellular transport and degradation of ¹²⁵I-TC-AOM were determined by means of subcellular fractionation in sucrose and Nyoadenz gradients of cells that were incubated with labeled ligand in the presence and absence of vanadate.

EXPERIMENTAL PROCEDURES

Materials—Nyoadenz was obtained from Nycomed (Oslo, Norway). Asialoorosomucoid was purged as described before (Tolleshaug and Berg, 1980) and labeled with ¹²⁵I (Tolleshaug, 1981) or by covalent attachment of a radioiodinated tyramine-cellobiose aduct according to Pittman et al. (1983). The adduct of tyramine and cellobiose was a generous gift from Helge Tolleshaug (Nycomed). Collagenase (type I), enzyme substrates, and bovine serum albumin (fraction V) were from Sigma; and vanadate from BDH Chemicals Ltd. (Poole, United Kingdom). Na₂₃₂₅ was from the Radio-chemical Centre (Amersham, United Kingdom).

Animals and Cells—Isolated rat liver parenchymal cells were prepared essentially according to Seglen (1976) as described before (Berg and Iversen, 1976). Male Wistar rats (~200 g) were used. The animals were given food and water ad libitum. The cells were incubated in 8.0 g of NaCl, 0.4 g of KCl, 0.06 g of Na₃HPO₄, 2H₂O, 0.047 g of KH₂PO₄, 0.2 g of MgSO₄, 7H₂O, 9.52 g of HEPES, 0.29 g of CaCl₂: 2H₂O, 10 g of albumin, and water to 1000 ml (incubation medium). The pH was adjusted to 7.5 by the addition of NaOH. Osmolality was 300 mosm/liter. Cell viability was always above 90% (trypan blue exclusion test).

Cell Fractionation—Homogenization of cells, preparation of post-nuclear fractions, and cell fractionation by means of iscopic centrifugation in sucrose and Nyoadenz gradients were done as described previously (Berg et al., 1985; Kindberg et al., 1987b).

Analytical Procedures—Radioactivities were measured in a Kontron gamma-counter. β-Acetyleglucosaminidase was assayed according to Barrett (1972). The densities of the gradient fractions were obtained from the refractive indices (Richwood, 1983). ATP was measured with luciferin and luciferase (De Luca and McElroy, 1978).

Cell Incubations and Determination of Binding, Uptake, and Degradation of ¹²⁵I-TC-AOM—Cells were incubated in Erlenmeyer flasks in a shaking incubator. Cell concentrations were 8–12 × 10⁶/ml. Binding of ligand to cell-surface receptors was measured after incubation of cells at 4 °C for 90 min. Cells were separated from incubation medium by centrifugation through dibutyl phthalate (Tolleshaug et al., 1985). Unspecifically bound ligand was determined by adding 10 mM EGTA to the cells prior to centrifugation through oil. Uptake of ¹²⁵I-TC-AOM in cells (usually at 37 °C) was measured as radioactivity associated with the cells following centrifugation through dibutyl phthalate (Tolleshaug et al., 1985). Unspecific uptake was determined by adding 10 mM EGTA to the cells prior to the addition of ligand. Degradation of ligand was followed by measuring radioactivity soluble in 10% trichloroacetic acid (Tolleshaug et al., 1985). To follow internalization of surface-bound ligand, the cells were first incubated at 4 °C with 50 nM labeled ligand for 60 min; the cells were then washed in incubation medium and incubated at 37 °C without extracellular ligand for various times. After the start of the incubation at 37 °C, cell samples were removed and mixed with equal volumes of medium with or without EGTA (final concentration of 10 mM). Cell surface-bound radioactivity is released by EGTA, and radioactivity remaining with the cells is internalized ligand. To measure retroendocytosed ligand, cells that had been incubated at 4 °C for 60 min with ¹²⁵I-TC-AOM were washed and then incubated at 37 °C for 7 min to internalize ligand. The cells were then washed in medium containing 10 mM EGTA and finally incubated at 37 °C in the presence and absence of 10 mM EGTA. During the final incubation at 37 °C, retroendocytosed ligand is released to the medium by EGTA. Washing of cells was usually done by adding 10 volumes of ice-cold incubation medium to 1 volume of cell suspension. The cells were then sedimented by centrifugation (50 × g, 1 min) and resuspended in cold incubation medium twice. It is essential that the cells are rapidly chilled to prevent a reduction in the amount of active cell-surface receptors (Weigel and Oka, 1983).

RESULTS

Effects of Vanadate on Binding of ¹²⁵I-TC-AOM—Suspensions of hepatocytes were incubated at 37 °C for 30 min in the presence of various concentrations of vanadate. The cell suspensions were then chilled by adding ice-cold incubation medium (9 ml of incubation medium/ml of cell suspension) and following sedimentation at low speed, the cells were resuspended in new medium and incubated at 4 °C in the presence of ¹²⁵I-TC-AOM. After 90 min, cell-surface binding was measured. The results (Fig. 1) show that surface binding was reduced to ~30% of control values in the presence of 10 mM vanadate.

To determine the rate at which vanadate reduced binding, hepatocytes were incubated at 37 °C in the presence of 10 mM vanadate for various times; and then, after washing in ice-cold incubation medium, binding of ¹²⁵I-TC-AOM was measured after incubation with ligand for 90 min. The results presented in Fig. 2 indicate that binding to cell-surface receptors was reduced with a half-time of ~3 min. This corresponds to the rate of internalization of cell-surface-bound ligand (Tolleshaug, 1981).

Vanadate Does Not Change Rate of Internalization of Ligand—To assess the effect of vanadate on the rate of internalization of surface-bound ligand, cells were first incubated in the presence of ¹²⁵I-TC-AOM at 4 °C for 90 min and then, after washing, were incubated at 37 °C in the presence and absence of vanadate (10 mM). The proportion of ligand internalized was measured after adding EGTA (10 mM) to release...
Vanadate Inhibits Endocytosis

FIG. 1. Effects of vanadate on cell-surface receptor content. Cell suspensions were incubated with the indicated concentrations of vanadate for 30 min at 37 °C and then, after washing, were incubated at 4 °C with 50 nM 125I-TC-AOM. After 90 min, cell surface-bound radioactivity was determined as described under "Experimental Procedures." Control cells bound 550 fmol of ligand/10^6 cells.

FIG. 2. Rate of reduction in cell-surface receptor content after adding vanadate. Cell suspensions were incubated with 10 mM vanadate at 37 °C. At the indicated times, cell samples were removed, washed, and incubated at 4 °C with 50 nM 125I-TC-AOM. After 90 min, cell surface-bound radioactivity was determined as described under "Experimental Procedures." Control cells (before the addition of vanadate) bound 515 fmol of ligand/10^6 cells.

FIG. 3. Rate of internalization of 125I-TC-AOM is not affected by vanadate. Cell suspensions were incubated for 60 min at 4 °C with 50 nM 125I-TC-AOM. The cells were then washed and incubated at 37 °C in the presence (●) and absence (○) of 10 mM vanadate. At the indicated times, cell aliquots were removed and treated with 10 mM EGTA to remove cell surface-bound ligand. Cell-associated radioactivities are presented as percent of that present in the cells at the start of the incubation at 37 °C (613 fmol of ligand/10^6 cells).

FIG. 4. Vanadate prevents reappearance of temperature-sensitive (state II) asialoglycoprotein receptors and does not affect activities of temperature-insensitive (state I) asialoglycoprotein receptors. The results shown were obtained with cells that had been incubated at 15 °C for 60 min to reduce the surface receptor content according to Weigel and Oka (1983). These cells contained state II receptors. At time 0, the cells were transferred to 37 °C and incubated further in the presence (●) and absence (○) of 10 mM vanadate. At the indicated time points, cell aliquots were removed, washed, and incubated at 4 °C with 50 nM 125I-TC-AOM. After 90 min, cell surface-bound radioactivity was determined as described under "Experimental Procedures." After 30 min at 37 °C, control cells bound 513 fmol of ligand/10^6 cells, and vanadate-treated cells bound 227 fmol of ligand/10^6 cells.

Vanadate Acts Selectively on State II Receptors—It has been demonstrated that the number of active cell-surface asialoglycoprotein receptors can be modulated by varying the incubation temperature. Thus, if the incubation temperature is gradually reduced from 37 °C to 20 °C (or less), the number of asialoglycoprotein receptors is reduced by ~50–70% (Weigel, 1987; Weigeland Oka, 1983). It has been shown that the receptors that remain active following temperature reduction differ from those that are regulable, and the two types of receptors have been called "state I" and "state II" receptors (Weigel, 1987), respectively. The results shown in Fig. 1 were obtained with cells expressing both state I and state II receptors. To determine whether vanadate reduced binding to state I receptors or if only state II receptors were affected, cells expressing both receptors were first incubated for 60 min at 15 °C to inactivate temperature-sensitive state II receptors. The cells were subsequently incubated at 37 °C in the presence and absence of 10 mM vanadate, and cell-surface binding was measured at various times after the start of the incubation at 37 °C. The results are shown in Fig. 4 and indicate that vanadate does not reduce state I receptors (closed circles). Furthermore, the drug prevented the increase in temperature-sensitive state II receptors. These data suggest that vanadate acts selectively on state II receptors.

Effect of Vanadate on Binding Is Reversible—When cells that had been treated with 10 mM vanadate at 37 °C were washed and reincubated at 37 °C, the number of surface receptors gradually increased to control levels during 15–30 min (Fig. 5).

Effect of Vanadate on Continuous Uptake of 125I-TC-AOM—The effect of various concentrations of vanadate on continu-
FIG. 5. Reversibility of effect of vanadate on cell-surface receptor content. Cell suspensions were incubated with 10 mM vanadate at 37 °C for 1, 2.5, 5, and 10 min. The cells were then washed and reincubated at 37 °C in the absence of vanadate. Samples were taken at the indicated times to determine the number of active surface asialoglycoprotein receptors (see "Experimental Procedures"). The cell-surface receptor content is presented as percent of that present on the cells at the start of the experiment (577 fmol of ligand/10⁶ cells).

FIG. 6. Effect of vanadate on rate of uptake of ¹²⁵I-TC-AOM. Cell suspensions were incubated at 37 °C with 60 nM ¹²⁵I-TC-AOM and with the indicated concentrations of vanadate. The cellular uptake of labeled ligand was measured after 30 min of incubation and is presented as percent of uptake in control cells. Uptake was determined as described under "Experimental Procedures." In control cells, uptake amounted to 63 fmol of ligand/10⁶ cells/min.

Vanadate Does Not Affect Retroendocytosis—Vanadate did not seem to affect the proportion of internalized ligand that was retroendocytosed. To measure retroendocytosis, cells were allowed to internalize ligand at 37 °C for 7 min, and they were subsequently washed at 0 °C in the presence of EGTA (10 mM) and finally incubated at 37 °C in the presence of EGTA to release ligand returning to the cell surface. Vanadate had no measurable effect on the amount of ligand released (Fig. 7).

FIG. 7. Retroendocytosis of ¹²⁵I-TC-AOM in presence (●) and absence (●) of 10 mM vanadate. Cell suspensions were incubated at 4 °C for 60 min with ¹²⁵I-TC-AOM and then washed and incubated at 37 °C for 7 min to internalize surface-bound ligand. The cells were then washed again in medium containing 10 mM EGTA and finally incubated at 37 °C with EGTA (10 mM) in the presence and absence of 10 mM vanadate. Labeled ligand released to the medium was measured at the indicated times and is presented as percent of radioactivity in the cells at the start of the incubation at 37 °C in EGTA-containing medium.

Effects of Vanadate on Degradation of ¹²⁵I-TC-AOM—To measure the effect of vanadate on the intracellular degradation of ligand independent of its effect on binding, vanadate was added to cells that had been given a pulse of ligand at 4 °C. Following washing, the cells were incubated at 37 °C with various concentrations of vanadate. The dose-response curve (Fig. 8) indicated that the maximal effect of vanadate was obtained at concentrations above 1 mM. The half-maximal effect of vanadate was seen at much lower concentrations of vanadate for degradation than for binding and uptake.

The effects of vanadate on degradation of ¹²⁵I-TC-AOM may be direct and/or indirect. Vanadate may act directly on the intralysosomal degradation, or it may interfere with the intracellular transport of endocytic vesicles to the site of degradation. To obtain preliminary information about the
Vanadate Inhibits Endocytosis

Vanadate inhibits degradation of long-lived/stable proteins labeled with [14C]leucine 24 h prior to the isolation of parenchymal cells. This finding was recently supported by studies done with L-132 human cells (Vargas et al., 1989).

Isolated parenchymal cells incubated in the type of medium used in this investigation have high autophagic activity. We have found previously that lysosomes of parenchymal cells incubated under step-down conditions (i.e. the medium described under "Experimental Procedures") gradually become more buoyant in isotonic Nycodenz gradients (Kindberg et al., 1987a) Fig. 11 indicates that during 90 min of incubation, the peak of β-acetylglucosaminidase in the Nycodenz gradients changed from 1.14 to 1.09 g/ml, indicating that the secondary lysosomes take up endogenous substrate by autophagy and form osmotic active particles that render the lysosomes more buoyant in the isotonic Nycodenz gradients. Fig. 11 shows that the addition of vanadate to the cells completely prevented the change in buoyant density.

ATP Content in Vanadate-treated Cells—Some of the changes observed after the addition of vanadate could conceivably be due to reduction in ATP levels. However, measurements of ATP concentrations showed clearly that they were stable during incubation with 10 mM vanadate up to at least 60 min (25.8 nmol/10⁶ cells at the start of the incubation; 27.7 nmol/10⁶ cells after 60 min incubation with vanadate).

DISCUSSION

The results obtained in this study indicate that vanadate affects at least two steps in receptor-mediated endocytosis of asialoglycoprotein in rat liver parenchymal cells in suspension: it reduces the number of active surface asialoglycoprotein receptors, and it inhibits the transfer of ligand from an endosomal compartment to the secondary lysosomes.

The vanadate treatment seemed to affect the surface receptors that are also sensitive to changes in incubation temperature (state II receptors) (Wang and Choppin, 1981). Vanadate had no effect on surface binding of ligand in cells expressing only state I receptors. The rate at which vanadate reduced surface binding suggested that it allowed internalization of one surface pool of state II receptors. These receptors did not return to the cell surface in an active state. As vanadate interferes with phosphoryl transfer and release reactions, it is tempting to speculate that the reduction in active surface asialoglycoprotein receptors is due to an effect of vanadate on the phosphorylation/dephosphorylation of the asialoglycoprotein receptor. This receptor contains threonine and serine residues in its cytoplasmic end that can be phosphorylated (Schwartz, 1984). Increased internalization of epidermal growth factor (Sibley et al., 1987), transferrin (Klauser et al., 1984), and asialoglycoprotein (Fallon and Schwartz, 1986, 1988) receptors has been observed after treatment of cells with phorbol esters. These data suggest a model in which receptors are phosphorylated on the cell surface, leading to internalization into compartment(s) where dephosphorylation can occur. Vanadate could, for instance, inhibit a phosphatase necessary for dephosphorylation and thereby inactivate/trap the receptors intracellularly. Vanadate may not inhibit thr/ser-phosphoryl phosphatase, and its effect therefore had to be indirect. It has been shown, however, that vanadate, similar to insulin, reduced phosphorylation of the IGF-II receptors which are phosphorylated on Ser/Thr residues (Kadota et al., 1987).

Subcellular fractionation experiments showed that vanadate inhibited degradation of ¹²⁵I-TC-AOM because it prevented its access to the lysosomes. The limited degradation

Fig. 9. Vanadate added up to 30 min after start of internalization of ligand inhibits ligand degradation. Cells that had been incubated at 4 °C with 50 nM ¹²⁵I-TC-AOM for 60 min were washed and subsequently incubated at 37 °C. Vanadate (10 mM) was added to cell samples after 0 (line E), 10 (line D), 15 (line C), and 30 (line B) min. Line A, control cells. Degradation of ¹²⁵I-TC-AOM was followed by measuring acid-soluble radioactivities as described under "Experimental Procedures." Radioactivities are presented as percent of total cell-associated radioactivity (700 fmo1 of ligand/10⁶ cells).

Fig. 10. P-acetylglucosaminidase. In control cells, the labeled degradation products formed coincided in the gradient with β-acetylglucosaminidase. In control cells, the labeled degradation products formed coincided in the gradient with β-acetylglucosaminidase.

Vanadate May Inhibit Formation of Autophagic Vacuoles—

Step(s) in the endocytic process at which vanadate acted, cell suspensions that had been given a pulse of ¹²⁵I-TC-AOM at 4 °C were subsequently incubated at 37 °C in the absence of ligand, and vanadate (10 mM) was added to the cells at various times after the start of the incubation. It was found that vanadate inhibited degradation almost instantaneously even if it was added 30 min after the start of the incubation at 37 °C (Fig. 9). This means that vanadate inhibits degradation by interfering at a relatively late step in the intracellular transport of ligand. It could inhibit transport of ligand from a late endosome to the lysosomes and/or intralysosomal degradation.

Intracellular Transport Studied by Subcellular Fractionation—To obtain further information about the effect of vanadate on the intracellular transport of endocytosed ligand, the subcellular distribution of ligand was followed by cell fractionation in sucrose gradients. Cells were given a pulse of ¹²⁵I-TC-AOM at 4 °C and were then washed and incubated at 37 °C in the presence and absence of 10 mM vanadate. Aliquots of cells were removed at various times, and postnuclear fractions were fractionated by sucrose gradient centrifugation.

By comparing the subcellular distribution of degraded and undegraded ligand with that of a lysosomal enzyme, β-acetylglucosaminidase, it could be concluded that vanadate completely inhibited the transfer of ligand to the secondary lysosomes (Fig. 10). The limited amount of degradation products that were found in the presence of vanadate did not accumulate in the same region of the gradient as the lysosomal marker β-acetylglucosaminidase. In control cells, the labeled degradation products formed coincided in the gradient with β-acetylglucosaminidase.

Seglen and Gordon (1981) found that vanadate inhibits degradation of long-lived/stable proteins labeled with [¹⁴C]leucine 24 h prior to the isolation of parenchymal cells. This finding was recently supported by studies done with L-132 human cells (Vargas et al., 1989).
Vanadate Inhibits Endocytosis

9004 Vanadate Inhibits Endocytosis

Density (g/ml)

Density (g/ml)

Fig. 10. Distribution of degraded (○) and undegraded (●) \(^{125}\)I-TC-AOM and \(\beta\)-acetylglucosaminidase (□) in sucrose gradients. Cells were incubated at 4 °C with 50 nM \(^{125}\)I-TC-AOM for 60 min and then, after washing, were incubated at 37 °C with or without 10 mM vanadate. After 90 min, samples of cell suspensions (50 x 10⁶ cells) were washed, and a postnuclear fraction was prepared as described (Berg et al., 1985) and applied to the sucrose gradients. Following centrifugation at 85,000 x g for 4 h, the gradients were fractionated and assayed for radioactivity, density, and enzyme activity. Enzyme activity and radioactivities in each fraction are presented as percent of total recovered activity in the gradient. The three upper fractions contain material not entering the gradient itself. At the start of the incubation at 37 °C, the cells contained 650 fmol of ligand/10⁶ cells.

Density (g/ml)

Density (g/ml)

Fig. 11. Distribution of degraded (○) and undegraded (●) \(^{125}\)I-TC-AOM and \(\beta\)-acetylglucosaminidase (□) in Nycodenz gradients. Cells were incubated at 4 °C with 50 nM \(^{125}\)I-TC-AOM for 60 min and then, after washing, were incubated at 37 °C with or without 10 mM vanadate. After 90 min, samples of cell suspensions (50 x 10⁶ cells) were washed, and postnuclear fractions were prepared as described (Berg et al., 1985) and applied to the Nycodenz gradients. Following centrifugation at 85,000 x g for 45 min, the gradients were fractionated and assayed for radioactivities, densities, and enzyme activity. Radioactivities and enzyme activity in each fraction are presented as percent of total recovered activity in the gradient. The three upper fractions contain material not entering the gradient itself. The distribution of \(\beta\)-acetylglucosaminidase for cells fractionated 15 min after the start of the incubation at 37 °C is shown (● - - ●). The cells contained 650 fmol of ligand/10⁶ cells at the start of the incubation at 37 °C.

of \(^{125}\)I-TC-AOM in the presence of vanadate does not take place in the secondary lysosomes as the degradation product, \(^{125}\)I-TC, accumulates in an organelle banding at a lower density in the gradients than the lysosomal markers.

We do not yet know the exact step at which vanadate interferes with the intracellular transport of \(^{125}\)I-TC-AOM. Vanadate inhibited degradation of \(^{125}\)I-TC-AOM given as a pulse at 4 °C even if it was added 30 min following the start of ligand uptake. At this time point, all ligand must have passed both the peripheral endosomes and the compartment for uncoupling of receptor and ligand. It is therefore reasonable to assume that the drug acts at a late transport step, i.e. between late endosomes (multivesicular bodies) and lysosomes. In addition, vanadate inhibits lysosomal degradation as such (Pillai and Zull, 1985; Seglen and Gordon, 1981) and may therefore inhibit ligand degradation if added long enough after the start of ligand uptake. Fractionation experiments showed, however, that when vanadate was present at the start of the uptake, it completely prevented uptake in the lysosomes.

The effect of vanadate on the intracellular transport of ligand resembles that of colchicine, which also inhibits transfer from a prelysosomal to a lysosomal compartment in rat liver parenchymal cells in suspension (Berg et al., 1985; Kolset et al., 1979; Oka and Weigel, 1983; Wolkoff et al., 1984). Both inhibitors may act as microtubular poisons, it has been demonstrated that vanadate alters the distribution of 10-nm filaments and separates them from microtubules (Wang and Choppin, 1981). At the same time, the distribution of organelles such as lysosomes is altered. The 10-nm filaments and microtubules may act together in the movement of cell organelles. The effect of vanadate could be related to that observed on the dynein Mg²⁺-ATPase (Gibbons et al., 1988). By inhibiting this enzyme, vanadate prevents the sliding of microtubules in cilia.
These data also confirm that vanadate inhibits cellular autophagy. This effect of vanadate could be revealed because autophagy in rat liver parenchymal cells in suspension renders the lysosomes less dense in isotonic Nycodenz gradients. Our data show that this density shift is completely prevented in cells treated with vanadate. Gordon and Seglen (1988) have shown that the heterophagic and the autophagic pathways meet prior to the lysosomal stage. Vanadate may therefore affect a prelysosomal step common to the heterophagic and the autophagic pathways.

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