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THE INFLUENCE OF CELLULAR ATP LEVELS ON RECEPTOR-MEDIATED ENDOCYTOSIS AND DEGRADATION OF ASIALO-GLYCOPROTEINS IN SUSPENDED HEPATOCYTES

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Abstract—Receptor-mediated endocytosis in suspended hepatocytes was studied in conjunction with ATP levels of the cells, which were decreased by the use of metabolic inhibitors. The receptor system studied was the asialo-glycoprotein receptor, and multiple aspects of the endocytic pathway were examined: binding of ligand, internalization, intracellular transport and proteolysis.

(1) Moderate concentrations of the inhibitors (e.g. 30 μM rotenone or 200 μM iodoacetamide) produced only a transient decline in the ATP levels of the cells. Two to four times higher concentrations reduced the ATP levels to about 1/10 of control cells.

(2) At low levels of ATP (less than 30% of controls) the uptake ceased completely after 10–20 min. Moderate reductions brought about by rotenone reduced the uptake roughly in proportion to the ATP levels; iodoacetamide and sodium fluoride had little influence on the energy production by the cells, but the rate of asialo-glycoprotein uptake was reduced to a small fraction of controls.

(3) The effect of rotenone on the rate of uptake was mainly due to a lower rate of internalization of occupied receptors; the half-time for internalization of surface-bound ligand was increased from 2.9 to 6.2 min in the presence of 42 μM rotenone. The binding capacity of the cell surface was also somewhat lower.

(4) There was no degradation of the asialo-glycoproteins which were taken up by cells treated with high concentrations of rotenone or iodoacetamide. This was shown to be due to a low rate of transport of the endocytosed protein into those endosomes (at density 1.15 g/ml in a sucrose gradient) which were delivering their contents to the lysosomes; coincidentally, there was an accumulation of ligand in light endosomes (density 1.11 g/ml), in which the ligand appears immediately after endocytosis.

Metabolic inhibitors which reduce ATP levels inhibit various types of endocytosis: fluid endocytosis [1], phagocytosis [2–5], and adsorbtive endocytosis [6]. Endocytosis plays an important role in the functioning of vital organs such as the liver and the kidneys. Reduced ATP levels inhibit renal reabsorbtion of proteins [7], probably by interfering with intracellular transport [8].

As regards receptor-mediated endocytosis, which is a multi-step process, it is likely that the internalization step is energy-requiring, because it involves fusion between cellular membranes. The transport of the internalized ligand molecules is also likely to be energy-dependent; the vectorial transport of vesicles probably requires the cooperation between contractile proteins and the cytoskeleton [9]. The processing of the endocytic vesicle [10] may require ion gradients, particularly pH gradients if dissociation of the ligand from its receptor is to occur [11, 12]. Receptor recycling, the path of which may lead through the Golgi region, is disrupted by the ionophore monensin [13, 14], indicating that ion gradients may be necessary. Finally, it is also likely that the intralysosomal degradation of the ligand requires a supply of energy, as *in vitro* studies have shown that ATP is necessary for the maintenance of low intralysosomal pH [15, 16].

In the present study, we have endeavoured to determine the effect of reduced ATP production on some of the steps involved in receptor-mediated endocytosis: internalization, intracellular transport, endosome–lysosome fusion, lysosomal degradation. We used total cellular ATP as a measure of the energy level of the cell; cytosolic and mitochondrial ATP concentrations are tightly coupled [17].

Endocytosis of asialo-glycoproteins in liver cells has been thoroughly studied [18]. The kinetics of the uptake [19] as well as the degradation [20] are simple. An important advantage of the system is that surface-bound ligand may be removed nearly instantaneously by the addition of a calcium chelator to the medium [19]. This treatment inactivates the cell-surface receptors, so that surface-bound and internalized ligands may be determined separately, and estimates of the rate of internalization may be made.

After homogenization of the hepatocytes, the distribution of labeled ligand between various types of membrane-bound vesicles may be determined by fractionation in a sucrose gradient [10, 21, 22]. The bulk of the labeled ligand molecules is recovered at a density of 1.15 g/ml. The ligand molecules in this

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peak are contained in endosomes, and they are slowly being delivered to the lysosomes [10, 22], which are recovered at a density of 1.20 g/ml in sucrose gradients. Thus, studying the uptake into suspended cells in conjunction with subcellular fractionation enabled us to trace the path of the ligand from the cell surface to the lysosomes.

Initially, we measured uptake and degradation of ^{125}I-asialofetuin along with ATP levels in hepatocytes incubated with several metabolic inhibitors. These studies revealed that rotenone in low concentrations had marked effects on ATP levels as well as endocytosis. This inhibitor was selected for a more detailed study, using subcellular fractionation in a sucrose gradient to determine the affected steps. The data indicated that reduced ATP production inhibited the processing of endocytic vesicles as well as recycling of the receptor.

METHODS AND MATERIALS

Protein ligands. Orosomucoid was obtained from Calbiochem-Behring Corp., Loewengraben 14, CH-6000 Lucerne, Switzerland, and fetuin ("Type III") from Sigma Chemical Co. (St. Louis, MO). The glycoproteins were desialylated by treatment with neuraminidase [19] and labelled with ^{125}I by a modification [19] of the sodium oxychloride method of Redshaw and Lynch [23].

Cells. Liver cells were prepared by perfusing the liver successively with calcium-free buffer and collagenase solution [24, 25]. Nonparenchymal cells were removed by differential centrifugation [26]. The cells were incubated in a minimal salt medium containing 1% bovine serum albumin on a shaking water bath at 37° [26]. No low molecular weight substrate was added as an energy source. The viability at the beginning of the incubation was 95% or better, as determined by the trypan blue exclusion method. If the viability fell below 70%, the results of the experiment were discarded.

Determination of cell-associated and acid-soluble radioactivity. Hepatocytes were separated from the medium by placing 250 μl of cell suspension on top of 200 μl of dibutyl phthalate in a narrow test tube and centrifuging in a table centrifuge for 30 sec at 3500 *g*. Only hepatocytes that are impermeable to trypan blue pass through the oil, so that only ligand associated with intact cells was included in the uptake measurements. The amount of radioactivity found after centrifuging through oil is referred to as *cell-associated* radioactivity. It includes surface-bound and internalized ligand (but no degradation products, see below).

Asialo-glycoproteins which are bound by receptors on the cell surface may be removed within 15 min at 4° by adding the calcium chelator EGTA [ethylene glycol bis-(2-aminoethyl ether)-*N*,*N*′-tetraacetic acid] to the cell suspension [19, 27]. The amount of *internalized ligand* was determined by adding the cell suspension to an ice-cooled test-tube containing 10 μl of 0.2 M EGTA. The tubes were left on ice and shaken occasionally before 200 μl of dibutyl phthalate was added. The tubes were centrifuged in as described above. The difference between the radioactivity in ordinary and EGTA-treated samples gives the amount of *surface-bound radioactivity*.

Internalization of surface-bound ligand is very fast (half-time of 3 min [19]). There is a measurable amount of surface-bound ligand only if there is extracellular ligand present to continuously replenish the pool of surface-bound ligand.

The amount of acid-soluble *degradation products* was determined by mixing 250 μl of cell suspension with an equal volume of 10% (w/v) of trichloroacetic acid or a 4% (w/v) solution of phosphotungstic acid in 2 N HCl according to whether the ligand was asialofetuin or asialo-orosomucoid, respectively. There is negligible degradation in medium from which the cells have been removed [26]. As the degradation products leave the cells very quickly [10], a measure of *the total uptake* of asialo-glycoproteins up to a certain time point is obtained by adding the amounts of cell-associated and acid-soluble radioactivity at that time point.

In control cells (20 min after the addition of ^{125}I-asialofetuin) typical values would be 12% surface-bound, 66% internalized, and 11% degraded of the total amount of ligand added. These values would add up to 78% cell-associated (surface-bound plus internalized) or 89% total uptake (cell-associated plus degraded).

Fractionation by isopycnic centrifuging. Briefly, the hepatocytes were homogenized in a Dounce homogenizer, the nuclear fraction was removed by centrifuging, and an aliquot of the cytoplasmic extract was placed on top of a sucrose gradient which was centrifuged until density equilibrium was attained [10, 21, 22].

Measurement of ATP concentrations. The amounts of ATP in the cells were determined by the use of a purified preparation of firefly luciferase [28] which was obtained from LKB-Wallac (Bromma, Sweden). The levels of the emitted light, which remained stable for several minutes, were determined in a LKB-Wallac Luminometer 1250. Standards solutions with ATP concentrations between 0.1 and 10 μM ATP were used; the standard curve was a straight line. The sensivitity of the assay was more than adequate for the present purpose.

Fifty microlitres of cell suspension was squirted into 1 ml of ice-cold 0.2 M perchloric acid. The precipitate was removed by centrifuging for 10 min at 2500 *g*. Two hundred microlitres of the supernatant was diluted with 800 μl of 0.1 M Tris–acetate buffer, pH 7.75, containing 5 mM EDTA. Fifty microlitres of this dilution was added to a mixture of 200 μl of the reconstituted luciferase reagent and 800 μl of dilution buffer in a clear plastic test-tube. The emitted light was determined in the luminometer.

RESULTS

ATP levels in suspended hepatocytes

Preliminary experiments revealed that the ATP level was close to zero in cells which had been left on ice for 15 min or more. Evidently, the production of ATP declines faster than its utilization as the cells are cooled. It was further found that the ATP concentration in the hepatocytes was re-established at a stable level after about 15 min of incubation at

Table 1. Uptake of asialo-glycoproteins and ATP levels in suspended hepatocytes—effects of metabolic inhibitors

Compound	Concentration (mM)	Pre-incubation (min)	Uptake* (% of controls)	ATP level†
Deoxyglucose	50	60	87	81
Iodoacetamide	0.1	10	100	114
	0.2	10	87	103
	0.4	10	17	100
Na-fluoride	3	60	101	105
	5	60	65	105
	10	60‡	58	108
Dinitrophenol	0.2	20	90	N.D.§
	0.2	60	79	60
	0.5	60	7	5
Na-azide	1	60	94	62
	3	60	53	19
	10	60	19	N.D.
	10	20	20	N.D.
Rotenone	0.01	15	71	73
	0.03	15	41	50
	0.1	15	16	37
Fructose	10	15	59	37

After the pre-incubation (column 3) at 37°, the cell suspensions were made 10 nM with respect to ^{125}I-asialo-fetuin.

* Uptake after 5 and after 10 min were expressed as percentage of the uptake in control cells; these percentages were averaged. The standard deviation between different experiments (N = 3–5) was about 20% of the stated values.

† Determined at the end of the pre-incubation.

‡ After the pre-incubation, the cells were oblong, with numerous blebs.

§ N.D., not determined.

37°. This ATP level, which remained stable for at least 2 hr in untreated cells, was about 15 nmoles per 10^6 cells. In comparison, the ATP concentration in rat liver is about 2.5 μmoles/g liver [29]. One gram of liver contains about 125×10^6 hepatocytes and 65×10^6 nonparenchymal cells [30]. If one assumes that the fraction of total ATP in the nonparenchymal cells is proportional to their volume, then the ATP concentration in hepatocytes *in situ* is about 18 nmoles/10^6 cells.

Effects of different metabolic inhibitors on ATP levels and uptake of ^{125}I-asialo-fetuin in suspended hepatocytes

The fact that cooling of the cells led to reduced ATP levels required us to pre-incubate the cells at 37° before the addition of the inhibitor. The duration of the pre-incubation was routinely 15–30 min.

Table 1 gives a survey of a series of experiments in which uptake of asialofetuin together with ATP levels were determined in hepatocytes which had been incubated with the inhibitors before the addition of ligand (10 nM ^{125}I-asialo-fetuin). Untreated cells took up nearly all of the labeled ligand after 20 min at 37°. Therefore, in order to make valid comparisons of uptake values, it was necessary to measure the uptake after 5 and 10 min, when typical uptake levels were 40–60% and 70–80%, respectively. The ATP levels presented in Table 1 were determined immediately following the pre-incubation with the inhibitor, and are representative for the period during which the uptake was measured (lower panel in Fig. 1).

The data in Table 1 show that rotenone, sodium azide, fructose, and dinitrophenol all affected both ATP levels and asialo-fetuin uptake. For instance, incubation of cells for 15 min with 0.1 mM rotenone reduced the uptake of asialo-fetuin to 16% and the ATP concentration to 37% of control values. Rotenone, sodium azide, and dinitrophenol are inhibitors of mitochondrial ATP production. Negligible effects on ATP levels were observed with low concentrations of three inhibitors of glycolysis, namely deoxyglucose, iodoacetamide, and sodium fluoride. Nevertheless, both iodoacetamide (0.4 mM) and fluoride (10 mM) had large effects on the uptake of asialo-fetuin. Possibly, iodoacetamide and fluoride are inhibitors of other enzymes involved in endocytosis. In the case of iodoacetamide, inactivation of an essential non-enzyme protein is a third possibility [31, 32].

Conceivably, a high-energy intermediate of glycolysis may be the energy source for a step in the internalization process. However, this is unlikely, because fructose stimulates glycolysis [33, 34], while it reduces the rate of uptake of asialo-fetuin. This reduction is likely to be a direct consequence of the reduced ATP level which is observed on the addition

Table 2. Effect of metabolic inhibitors selectively on degradation

Compound	Concentration (mM)	Degradation (% of control)
Rotenone	0.025	71.2
	0.113	46.5
	0.252	43.4
Iodoacetamide	0.15	71.9
	0.25	42.7
	0.4	41.5
Sodium azide	5	39.7
Fructose	10	54.2
Sodium iodoacetate	0.2	83.5
	0.4	34.5

The cells (8×10^6 per ml) were incubated for 20 min with 25 nM asialo-orosomucoid at 37°, then the suspension was chilled, and the medium was changed. The suspension was divided into several equal portions, one of which served as the control (no addition); the others received inhibitors to the concentrations stated.

Duplicate samples for the determination of acid-soluble radioactivity were taken at least three times (at intervals of 10 min or more) over a 40-min period, and a straight line was placed through the points by the method of least squares. Finally, the slopes of the lines were compared.

of fructose to the cells. In high concentrations, fructose depresses ATP levels because ATP is required for its phosphorylation [34].

Effects of metabolic inhibitors on the degradation of asialo-fetuin

In order to study effects of metabolic inhibitors on the cellular degradation of asialo-fetuin (independent of their effects on uptake), the cells were first allowed to bind and internalize the labeled ligand at 37°. Then the cells were washed in order to remove free ligand, and degradation (production of acid-soluble radioactivity) was measured during a second incubation at 37° in the presence or absence of inhibitors. Degradation was followed for 60 min, allowing agents which do not act instantaneously to exert their full effect.

At comparable concentrations of the inhibitors, the effects specifically on degradation (Table 2) were equal to or smaller than the effects on uptake (Table 1). Rotenone in concentrations of about 0.1 mM inhibited uptake to 16% and degradation to 43% of control values. Very similar effects were seen with iodoacetamide (at higher concentrations).

Time course of the effects of rotenone, iodoacetamide, and fructose on the heterophagy of ^{125}I-asialo-fetuin

Rotenone (Fig. 1), iodoacetamide, and fructose had marked effects on ATP levels as well as uptake and degradation of asialo-fetuin. The changes with time in these parameters after the addition of each of the three inhibitors were studied in greater detail. The zero time levels in Fig. 1 correspond to the ATP levels presented in Table 1. It depicts the effects of three concentrations of rotenone on ATP levels

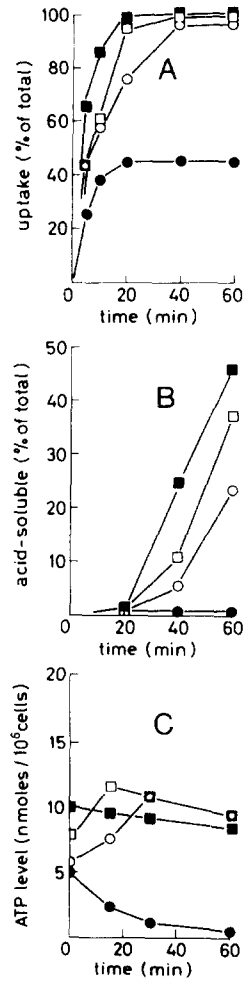

Fig. 1. Effects of rotenone on the uptake (panel A), degradation (B), and ATP levels (C) of suspended hepatocytes. The cells (8×10^6 per ml) were first pre-incubated with no additions for 15 min at 37°. Fifteen minutes before zero-time, 10 μM (□), 30 μM (○), or 100 μM (●) rotenone were added; no addition to controls (■). As the cells were pre-incubated with the inhibitors, the ATP levels were different at time zero. At zero-time, all of the flasks were made 10 nM with respect to ^{125}I-asialo-orosomucoid, and the amounts of cell-associated and acid-soluble radioactivity were determined at intervals. "Uptake" denotes the sum of acid-soluble and cell-associated radioactivity. Degradation in the medium is negligible [26].

(panel C), as well as uptake (A) and degradation (B) of asialo-fetuin. The presence of small concentrations of rotenone (below 30 μM) caused ATP levels to decline for several minutes, but then the levels increased, reaching a value that was somewhat higher than in the control cells. This "rebound" effect was reproducible in a qualitative sense. Figure 1 shows that ATP levels kept declining in the presence of 0.1 mM rotenone; after 60 min, the cells were virtually depleted of ATP.

A similar pattern was observed for high concentrations of iodoacetamide (not shown), but not for fructose; 10 mM fructose caused a stable

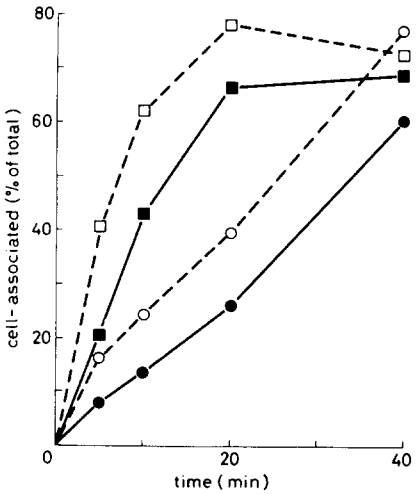

Fig. 2. Effect of rotenone on the rate of internalization of surface-bound asialo-glycoprotein. One cell suspension (8×10^6 cells per ml) was made 42 μM with respect to retenone 2 min before ^{125}I-asialo-fetuin (20 nM) was added. Only ^{125}I-asialo-fetuin (20 nM) was added to the control suspension. In addition to the usual uptake samples, which give the sum of internalized and surface-bound ligand, 600 μl aliquots were removed simultaneously. These aliquots were poured into chilled tubes containing 15 μl of 0.2 M EGTA (final concentration, 3 mM). The EGTA-treated cells were briefly warmed to 37° before uptake samples were taken in the usual manner. These samples measure only internalized ligand [19, 27]. □, Total cell-associated radioactivity in control cells; ■, total cell-associated radioactivity in the rotenone-treated cells; ○, internalized ligand in control cells; and ●, internalized ligand in rotenone-treated cells.

reduction in ATP levels to about 40% of control levels. The data for uptake (Panel A in Fig. 1) show, as pointed out above, that virtually all of the labeled ligand was removed from the medium after 20 min by the control cells. In the presence of high concentrations of rotenone (0.1 mM) or iodoacetamide (0.4 mM) the uptake ceased after 20 min, although much intact ligand was still present in the medium. The reason was probably that decreased ATP levels reduced the rate of internalization of ligand (see Fig. 2 below).

The degradation curves in Fig. 1 indicate much lower degradation in the presence of rotenone than the data in Table 2. The reason for this discrepancy is that the values for degradation in Fig. 1 were obtained on cells which were treated with inhibitor before the addition of asialo-fetuin, so that the effect is on the uptake as well as on degradation of the ligand. A reduced uptake leads to reduced degradation, because only internalized ligand is degraded. On the other hand, in the experiments of Table 2, the cells were allowed to take up the same amount of ligand before the inhibitor was added, so that the rate of degradation could be compared without regard to variations in the amount of ligand available for degradation.

In uptake experiments, the curves for internalized and total cell-associated ligand were indistinguishable in the control cells (not shown). Even at the highest concentrations tested of iodoacetamide or fructose, the major part of the cell-associated ligand was internalized. In conjunction with the fact that high concentrations of these inhibitors nearly abolished degradation, this means that they act strongly on intracellular transport. This was confirmed by cell fractionation (see below). It is, however, interesting to note that in specifically designed experiments, a moderate concentration of rotenone may be shown to inhibit the internalization process (see below).

Effects of rotenone on the internalization of asialo-orosomucoid

Surface-bound and internalized asialo-orosomucoid were estimated by a method which is based on the observation that binding of asialo-glycoproteins to the hepatic receptor is calcium-dependent (see Materials and Methods). Figure 2 shows the results of an experiment in which binding as well as internalization of ^{125}I-asialo-orosomucoid were determined in cells incubated with or without rotenone. The half-life for internalization of surface-bound asialo-glycoprotein was estimated from measurements during that period when the uptake was close to a "steady state", i.e. when the amount of surface-bound ligand was approximately constant and the amount of internalized asialo-orosomucoid was increasing steadily. The rate of internalization was computed by dividing the rate of increase in the amount of internalized (EGTA-resistant) ligand by the amount of surface-bound (EGTA-releasable) ligand [19]. This method does not require assumptions about the total number of receptors on the cell surface, the percentage of occupied receptors etc. In the control cells, the half-life for internalization was 2.9 min, in good agreement with previous values [19, 35].

The maximum amount of surface-bound asialo-orosomucoid in the presence of 42 μM rotenone was about 70% of that in the control cells, a moderate reduction. Computation of the half-life for internalization in the treated cells yielded a value of 6.2 min. This values shows that the main cause of the low rate of uptake in the rotenone-treated cells is a reduced rate of internalization of surface-bound asialo-glycoprotein. The fact that the binding capacity of the cell surface is also reduced, suggests that the recirculation of receptors is less efficient.

Changes in the intracellular distribution

Even though both binding and internalization were somewhat reduced in the presence of rotenone, the marked inhibition in degradation could only be partially explained by these changes. The major reason for the reduced degradation might be that intracellular transport proceeds more slowly in the rotenone-treated cells. The affected step might be incorporation of the ligand into the "mature endosomes" which are recovered at a density of 1.15 g/ml in a sucrose gradient; it might be processing of these endosomes [10], or it might be endosome–lysosome fusion [21, 22, 36]. In order to test these possibilities, the cells were preincubated with

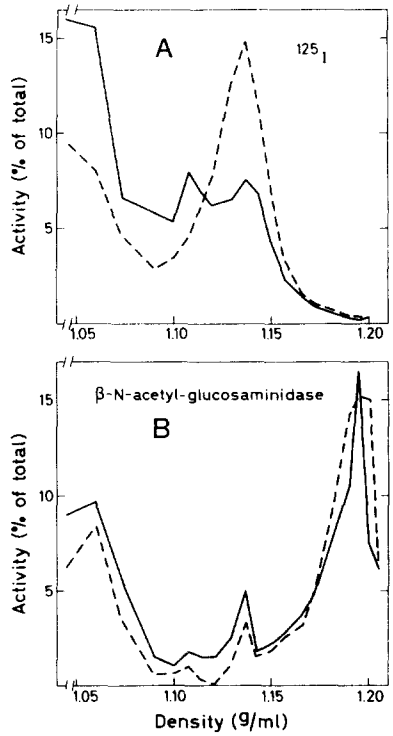

Fig. 3. Effect of rotenone on the intracellular distribution of ^{125}I-asialo-orosomucoid. Two flasks containing cell suspensions (10×10^6 cells per ml) were incubated for 15 min at 37°, then 100 μM rotenone was added to one of them, and the incubation was continued for another 15 min at 37°. Both flasks were made 0.5 nM with respect to ^{125}I-asialo-orosomucoid. The cells were incubated in the presence of ligand for 10 min, then the flasks were placed on ice. The cells were washed, homogenized and fractionated (see Methods). Distribution of ^{125}I in panel A, β-N-acetylglucosaminidase in panel B (marker enzyme for lysosomes) for control cells (– – –) and rotenone-treated cells (——), respectively.

0.1 mM rotenone, then labeled asialo-orosomucoid was added, and the incubation was continued for 15 min before fractionation of the cells.

In the distribution profiles from the treated cells as well as from the control cells, a distinct peak of "mature endosomes" appears at $d = 1.14$ g/ml (Fig. 3A). In the rotenone-treated cells only, there is an additional peak at $d = 1.11$ g/ml. About one-half of the label in the gradient is recovered in the latter peak.

The distributions of the lysosomal marker enzyme β-*N*-acetylglucosaminidase were very similar in the two gradients, nearly all of the activity being recovered between 1.19 and 1.20 g/ml (Fig. 3B). The distribution curves of 5′-nucleotidase activity (marker enzyme for the plasma membrane) showed a single peak at 1.16 g/ml (not shown) in control cells and in treated cells. No labelled protein in the gradient is bound to receptors on fragments of the plasma membrane, because the sucrose solutions contain EDTA in sufficient concentrations to inactivate the receptors by chelating calcium ions; this means that all of the radioactivity in the gradient is contained within membrane-bound vesicles.

DISCUSSION

The effects of metabolic inhibitors which have been observed, are: first, a decreased rate of internalization of surface-bound ligand; and second, a decreased number of receptors on the cell surface. In conjunction, these two effects suggest that the pathway of receptors recycling through the interior of the cell is impeded at some point. If the rate of internalization was decreased, while receptor recycling occurred normally, an accumulation of receptors on the cell surface would be expected.

An effect of rotenone on a step which follows immediately after internalization, was suggested by the observation that the inhibitor had only a moderate effect on degradation if it was added after internalization was complete (Table 2), but it reduced degradation dramatically if it was added before the ligand (Fig. 1B). In the latter case, the effect was not secondary to lower uptake, because the amount of cell-associated radioactivity remained at one-half of the control cells.

The interpretation that rotenone affects a step which follows immediately after internalization, was supported by cell fractionation studies. Treatment of the cells with rotenone caused an additional peak of "light endosomes" at $d = 1.11$ g/ml to appear in the distribution profile of radioactive ligand after fractionation on a sucrose gradient. There is some evidence that the "light endosomes" are the ones which are formed immediately after internalization [11, 14, 37]. The contents of these endosomes are not transferred directly to the lysosomes [27].

An intriguing finding was that the effects of rotenone and iodoacetamide on endocytosis were qualitatively similar, even though moderate concentrations of iodoacetamide did not reduce the cellular ATP levels; its effect on degradation may be due to irreversible inhibition of thiol enzymes, including lysosomal proteases. On the other hand, a specific inhibitor of thiol proteases, leupeptin, selectively inhibits degradation. It has no effect on the uptake of asialo-glycoproteins [22].

Metabolic inhibitors might exert their effects on internalization by decreasing the membrane potential. However, the uptake of asialo-glycoproteins in suspended hepatocytes is not affected on incubation of the cells in a medium in which all of the sodium ions have been replaced by potassium ions [38], a treatment which causes a marked reduction in the membrane potential [39]. Thus, a low membrane potential does not affect the uptake of ligand by the asialo-glycoprotein receptor.

The processing of endocytic vesicles probably involves transport of protons into these organelles [40, 41], and the resulting decrease in pH is a prerequisite for the dissociation of ligand from the receptor [11, 12, 41]. Rotenone and other metabolic inhibitors slow down the processing by reducing the supply of ATP needed for the active transport of protons. Interestingly, the inophore monensin has an effect on the equilibrium density of endocytic vesicles [14] which is identical to that of rotenone.

The mechanism of action of the two inhibitors could be similar, in that monensin, which catalyzes the exchange of sodium ions for protons across membranes, is likely to disrupt the proton gradient that maintains the acid pH inside the endocytic vesicle. In fact, Harford *et al.* found that the metabolic inhibitors azide and deoxyglucose (used in combination) inhibited the acidification of endocytic vesicles [11].

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