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Uptake of mannose-terminated glycoproteins in isolated rat liver cells

Evidence for receptor-mediated endocytosis in hepatocytes

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Even though most of the hepatic binding capacity for mannose-terminated glycoproteins has previously been shown to reside in the hepatocytes (not in the non-parenchymal cells), detailed evidence for the specific uptake of mannose-terminated ligands has been lacking. In the present studies, yeast invertase, a large glycoprotein (M_r 270000) containing about 50% mannose, was shown to be taken up into hepatocytes by receptor-mediated endocytosis. The uptake was saturable and could be specifically inhibited by mannosides or by a Ca^{2+} chelator. The asialo-glycoprotein receptor was not involved. The low-M_r (13000) ligand ribonuclease B, which contains a single high-mannose glycan, was not taken up by hepatocytes; however, it was taken up as fast as invertase by non-parenchymal liver cells. After injection of ^{131}I-invertase into a rat *in vivo*, about one-half of the labelled protein was recovered in the hepatocytes. On a per-cell basis, each endothelial cell contained 3–4 times as much radioactivity as did the hepatocytes. On fractionation of hepatocytes in sucrose gradients, invertase showed a different intracellular distribution from that of asialo-fetuin, in that invertase moved much faster into that region of the gradient where the lysosomes were recovered. This indicates that invertase and asialo-fetuin are not transported intracellularly by identical mechanisms.

The existence of a receptor that is specific for glycans terminating in mannose or *N*-acetylglucosamine has long been recognized. It has been shown to be active in the endocytosis of glycoproteins by alveolar macrophages (Stahl *et al.*, 1978) and by non-parenchymal liver cells (Steer & Clarenburg, 1979; Maynard & Baenziger, 1981). The binding activity of the receptor is Ca^{2+}-dependent (Stahl *et al.*, 1980; Warr, 1980). An autoradiographic study of the uptake of mannose-terminated orosomucoid derivatives *in vivo* showed that the endothelial cells of the liver were very active (Hubbard *et al.*, 1979).

It was shown that hepatocytes contain nearly all of the hepatic binding capacity for mannose- or *N*-acetylglucosamine-terminated glycoproteins (Maynard & Baenziger, 1982; Mori *et al.*, 1983). Even so, those authors could not show that these glycoproteins were actually endocytosed by the hepatocytes. The ligands that were used were a mannan of M_r 40000 from yeast (Mori *et al.*, 1983) or glycoproteins containing one high-mannose chain per molecule, and glycopeptides, which included the high-mannose chains (Maynard & Baenziger, 1982). Preliminary evidence for the uptake of a mannose-terminated orosomucoid derivative has been presented (Prieels *et al.*, 1979).

In the present studies, we used a ligand of very high M_r (270000) as well as a high content of mannose (about 50%). The mannose residues are contained in several large branched chains, which are attached to the peptide by way of two *N*-acetylglucosamine residues (Lehle, 1980). There are no galactose or *N*-acetylgalactosamine residues in the molecule, so that binding by the asialo-glycoprotein receptor may be rigidly excluded.

We also studied the uptake of a ligand whose properties in this system have been examined previously: bovine RNAase B (M_r 13000). It has one *N*-linked high mannose glycan. The uptake of this ligand was also measured in non-parenchymal liver cells, in order to compare the uptake system with that of hepatocytes.

Abbreviation used: RNAase, ribonuclease.

Experimental

Proteins

Invertase (β-fructofuranosidase, EC 3.2.1.26) from baker's yeast was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.; type VII, catalogue no. I-4504). To remove contaminating glycoproteins, the invertase was purified as described by Trimble & Maley (1977), except that the DEAE-cellulose chromatography was performed at room temperature. The protein content of the fractions was determined from the A_{280}, by taking the A_{280} of a 1 mg/ml solution as 2.25 (Trimble & Maley, 1977).

The invertase was labelled with ^{125}I or ^{131}I by the sodium oxychloride oxidation method of Redshaw & Lynch (1974). To 20 μl of a 1 mg/ml solution was added 5 μl of 0.5 M-sodium phosphate buffer, pH 7.4, then 40 MBq (0.5 mCi) of $Na^{125}I$ (or 80 MBq of $Na^{131}I$), followed by three additions of 5 μl of 1 mM-NaOCl solution at 30 s intervals, with careful shaking between each addition. The reaction was stopped by 250 μl of 0.05 M-KI in 0.05 M-phosphate buffer, pH 7.4, containing 1% human serum albumin. The preparation was dialysed against several changes of the phosphate buffer. The labelled protein contained one or two iodine atoms per molecule. It was stored at -20°C and used within 2 months.

RNAase B (type XIIB) was obtained from Sigma. Asialo-fetuin was prepared as described previously (Tolleshaug, 1981), and these proteins were also iodine-labelled by the sodium oxychloride oxidation method as described (Tolleshaug, 1981). Neuraminidase (type VIII) (sialidase, EC 3.2.1.18) and mannan from *Saccharomyces cerevisiae* were also purchased from Sigma.

Cells

Liver cells were prepared from male Wistar rats weighing 200–300 g by Seglen's (1976) modification of the collagenase perfusion method of Berry & Friend (1969). After the collagenase treatment, the cells were shaken loose into an ice-cold iso-osmotic buffer containing 2% bovine serum albumin (Nilsson & Berg, 1977). Non-parenchymal cells were removed from the hepatocyte preparation by differential centrifugation as described by Tolleshaug *et al.* (1977); the final suspensions contained less than 2% non-parenchymal cells. All cells were incubated for at least 15 min at 37°C before the ligands were added. They were suspended at a density near 10×10^6 cells/ml.

Preparation of non-parenchymal liver cells

First, the hepatocytes were either removed by differential centrifugation (Nilsson & Berg, 1977), or the hepatocytes were killed by incubation with the enterotoxin from *Clostridium perfringens* type A. The toxin was prepared by the method of Sakaguchi *et al.* (1973) as modified by Granum & Skjelkvaale (1977).

Before the incubation with enterotoxin, the suspension was diluted with 30% of iso-osmotic buffer (Nilsson & Berg, 1977). Then 0.8–1.0 mg of freeze-dried enterotoxin was added to the suspension (volume 80 ml), and the mixture was incubated at 37°C until the hepatocytes were permeable to Trypan Blue. If a longer time than 15 min was required, a further amount of enterotoxin was added (about 0.2 mg). When nearly all of the hepatocytes had been rendered permeable to Trypan Blue, the suspension was filtered through nylon mesh (100 μm), and centrifuged for 3.5 min at 4°C at 1750 rev./min in a Sorvall HB-4 rotor. The cell pellet was resuspended in 30 ml of 2% bovine serum albumin in the iso-osmotic buffer, and divided between two centrifuge tubes (2.5 cm diam. × 10 cm). The suspensions were mixed with metrizamide solution (40%, w/v) in the proportion of 1 vol. of metrizamide solution to 0.75 vol. of cell suspension. The solutions were thoroughly mixed, and covered with 1 ml of 2% albumin in iso-osmotic buffer solution. They were centrifuged for 3.5 min at 4000 rev./min (1500 *g*) in a refrigerated table centrifuge. The non-parenchymal cells (which are impermeable to metrizamide after toxin treatment, in contrast with the hepatocytes) were recovered at the interface between the metrizamide and the albumin solutions. They were removed with a Pasteur pipette, care being taken to minimize contamination with metrizamide solution. The cells from each centrifuge tube were placed in a 10 ml centrifuge tube (1.3 cm × 10 cm), diluted to 10 ml with 2% albumin in iso-osmotic buffer, and centrifuged for 3.5 min at 4000 rev./min in the HB-4 rotor. The pellets were resuspended in 2% albumin solution, centrifuged down and resuspended. Finally, the suspension was diluted to a suitable volume with 1% albumin in iso-osmotic buffer before the cells were counted in a haemocytometer.

Preparation of purified endothelial cells

After incubation of the initial cell suspension with *C. perfringens* enterotoxin to remove hepatocytes, endothelial cells were separated from Kupffer cells (liver macrophages) by centrifugal elutriation. The separation was performed at 15°C in a Beckman JE-6 elutriator rotor in a Beckman J-21 centrifuge. Some 20×10^6–100×10^6 non-parenchymal cells were introduced into the elutriation system at a flow rate of 11.3 ml/min and 2200 rev./min. After gradual increase of the flow rate to 25.4 ml/min, the endothelial cells flowed out of the elutriation chamber. They were concentrated by centrifugation at 1500 *g* for 4 min and resuspended.

Preparation of purified Kupffer cells (liver macrophages)

The initial liver cell suspension was treated with Pronase to remove the hepatocytes (Berg & Boman, 1973). Some 20×10^6–100×10^6 non-parenchymal cells were introduced into the elutriation chamber at a flow rate of 25.4ml/min and 2500rev./min. The Kupffer cells were washed out at a flow rate of 53.0ml/min, concentrated by centrifugation for 4min at 1500*g* and resuspended in a small volume of incubation buffer.

Determination of cell-associated and acid-soluble radioactivity

For determination of cell-associated radioactivity, a 250 μl portion of the cell suspension was placed on top of 200 μl of water-immiscible separation fluid in a narrow 500 μl test-tube. The tube was centrifuged at 4000*g* for 30s in a table centrifuge, and the tip containing the cell pellet was cut off. The separation fluid was dibutyl phthalate for hepatocytes, or, for non-parenchymal cells, a mixture of 1 vol. of dinonyl phthalate to 4 vol. of dibutyl phthalate. Non-specific cell-associated radioactivity was subtracted (determined by adding unlabelled ligand at 10 times the highest concentration of labelled ligand to a separate portion of the cell suspension).

For determination of acid-soluble radioactivity, a 250 μl sample of the cell suspension was mixed with a solution of trichloroacetic acid or phosphotungstic acid (sodium salt). RNAase B and yeast invertase are partially soluble in 5% trichloroacetic acid. In all of the experiments with these proteins, acid-soluble radioactivity was determined after precipitation with 2% phosphotungstic acid in 1 M-HCl (final concn.) and centrifuging for 10min at 4000*g*. The radioactive degradation products from iodine-labelled proteins in hepatocytes are almost exclusively iodide ion (Tolleshaug *et al.*, 1979). In non-parenchymal liver cells, substantial amounts of iodotyrosines are released (Nilsson & Berg, 1977). The increase in acid-soluble radioactivity was not significantly different whether intact proteins were precipitated with 10% trichloroacetic acid, 1% phosphotungstic acid/0.5M-HCl or 2% phosphotungstic acid/1 M-HCl (final concns.); only the zero-time values were different. These tests indicated that 2% phosphotungstic acid/1M-HCl could be used to determine acid-soluble radioactivity from non-parenchymal cells. In all experiments, non-specific degradation was determined by adding a 10-fold excess of unlabelled ligand to a separate sample of the cell suspension. The values for non-specific degradation were subtracted from the experimental values.

Subcellular fractionation by isopycnic centrifugation

Isopycnic centrifuging in a sucrose gradient was performed as described previously (Tolleshaug *et al.*, 1979; Berg & Tolleshaug, 1980). In outline, the procedure was as follows. The hepatocytes were washed in 0.25M-sucrose, homogenized in a Dounce homogenizer, the nuclear fraction was removed, and the supernatant was layered on top of a continuous linear sucrose gradient. After centrifuging, 20 fractions (2ml each) were collected. All of the sucrose solutions used for washing of the hepatocytes or forming the gradient contained 1 mM-EDTA and 1 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.2.

Results

Uptake of yeast invertase into liver cells of the intact rat

After injection of a trace amount of ^{131}I-labelled yeast invertase into a rat, 98% of the injected radioactivity was recovered in the liver. The liver was perfused and the cells were separated and fractionated. The endothelial cells contained more than half of the labelled glycoprotein, most of the remainder being present in the parenchymal cells (Table 1). When the recoveries in each cell type were re-computed on a per-cell basis, each endothelial cell was found to contain 3–4 times more invertase than did any other cell type, confirming previous reports that endothelial cells are very active in endocytosing mannose-terminated glycoproteins (Hubbard *et al.*, 1979). We found, however, that nearly half of the invertase was re-

Table 1. *Distribution of ^{131}I-invertase among liver cells in vivo*

^{131}I-invertase (1 μg) was injected into the right femoral vein of a 250g rat, and 13min later perfusion of the liver was started. Liver cells were separated and fractionated as described in the Experimental section; stellate cells were separated from the other non-parenchymal cells on a Percoll gradient (Blomhoff *et al.*, 1984). The recoveries in each cell fraction were corrected for contaminating cell types (but not for losses of radioactivity through degradation of the ligand during liver perfusion and cell purification). The number of cells in 1g of rat liver was taken as 125×10^6 hepatocytes and 60×10^6 non-parenchymal cells. Of the latter, 57% were endothelial cells, 30% Kupffer cells and 13% stellate (fat-storing) cells.

Cell type	Recovery (% of dose)	^{131}I-invertase (ng/10^6 cells)
Parenchymal	38	0.41
Kupffer	10	0.68
Endothelial	52	2.01
Stellate (fat-storing)	0.5	0.09

covered in the parenchymal cells, and that each parenchymal cell contained, on average, as much of this glycoprotein as did the Kupffer cells.

One source of error is the loss of radioactivity from the liver during the perfusion; subsequently, the cell-purification procedures entail incubating suspensions of non-parenchymal cells at 37°C (see the Experimental section), which causes further losses through degradation of intracellular invertase. As these losses occurred before the cells were purified, reliable corrections cannot be made. For this reason, the values in Table 1 may only be taken as a guide to the actual distribution of invertase between the cell types of the liver at the start of the perfusion. In any case, the main point is that, after injection *in vivo*, a very substantial fraction of the invertase was recovered in the parenchymal cells of the liver.

Uptake of mannose-terminated glycoproteins in suspended non-parenchymal liver cells

The kinetics of uptake of bovine RNAase B and yeast invertase were studied in suspensions of non-parenchymal cells, obtained by differential centrifugation immediately after collagenase treatment of the liver. The suspensions contained Kupffer cells (liver macrophages) as well as endothelial cells, and negligible numbers of hepatocytes.

At comparable concentrations of the two ligands, the number of cell-associated invertase molecules per cell (Fig. 1) was somewhat higher than the number of cell-associated RNAase molecules (Fig. 2). The uptake of both ligands showed saturability in the non-parenchymal cells. The uptake of invertase and RNAase B was also examined in suspensions of purified endothelial cells (Fig. 3). At the initial concentrations of the two ligands chosen in this experiment, the percentages of cell-associated ligand were about the same for RNAase B and invertase. The number of RNAase molecules taken up per cell per unit time was also roughly equal to the number of invertase molecules.

In these experiments (Figs. 1–3), the fraction of degraded ligand at the end of the incubation was only 3–5% of the amount initially added, and less than this percentage at concentrations higher than 1 nM.

Uptake of mannose-terminated glycoproteins in hepatocytes

The uptake of yeast invertase in hepatocytes was studied at concentrations of ligand in the range 0.06–70 nM (Fig. 4). The uptake was clearly saturable. The increase in the percentage of cell-associated ligand ceased after 10–20 min. Subsequently, the number of molecules that were taken up by the cells equalled the number of molecules that were broken down. Degradation of invertase continued at the same rate for 90 min (results not shown).

At roughly the same cell concentrations and concentrations of ligand, the uptakes of invertase into

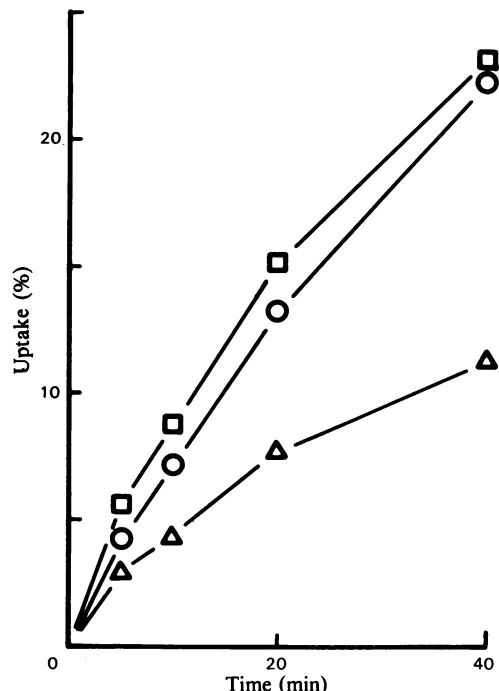


Fig. 1. *Endocytosis of yeast invertase in a suspension of mixed non-parenchymal liver cells*

Non-parenchymal liver cells were prepared by differential centrifugation. The final suspension contained 8×10^6 cells/ml (less than 2% hepatocytes). It was divided between four flasks which were made 0.1 nM (□), 1.0 nM (○) or 10 nM (△) with respect to ^{125}I-invertase. Specific degradation (determined by precipitation with phosphotungstic acid) was less than 2%.

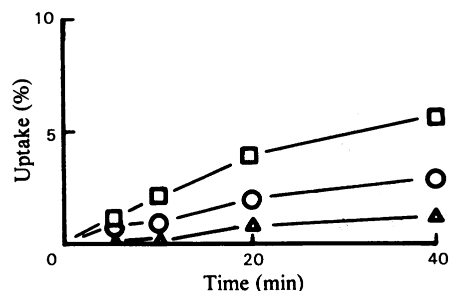


Fig. 2. *Endocytosis of RNAase B in a suspension of mixed non-parenchymal liver cells*

Experimental conditions were as in Fig. 1, except that the flasks were made 0.3 nM (□), 3.0 nM (○) or 30 nM (△) with respect to ^{125}I-RNAase B.

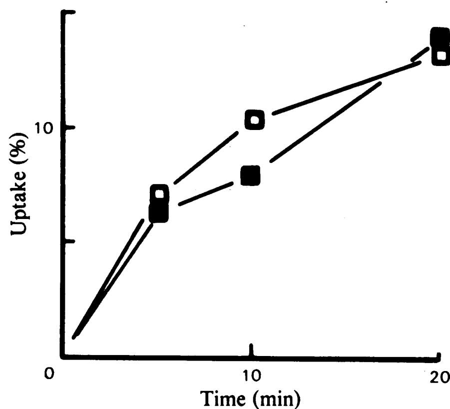


Fig. 3. *Endocytosis of yeast invertase and RNAase B in purified liver endothelial cells*
Endothelial cells were purified by treatment of the initial cell suspension with *C. perfringens* enterotoxin, followed by centrifugal elutriation. The final suspension contained 7×10^6 cells/ml (4% Kupffer cells, 6% stellate cells, no hepatocytes). Two different samples of the suspension were made 0.22nM with respect to ^{125}I-invertase (□) or 0.31mM with respect to ^{125}I-RNAase B (■). Specific degradation was less than 1% (determined by precipitation with phosphotungstic acid).

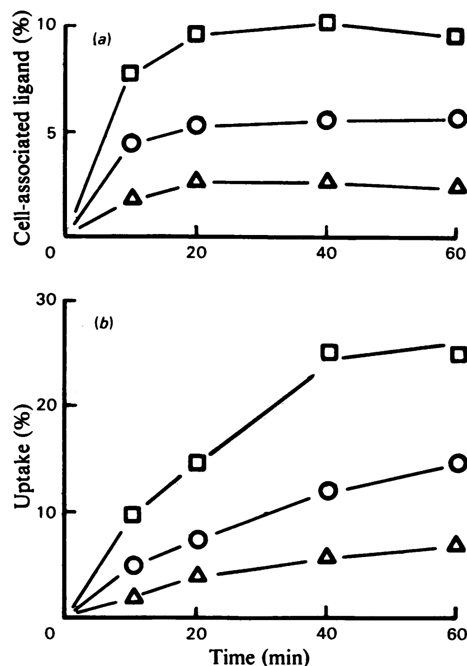


Fig. 4. *Endocytosis of yeast invertase in hepatocytes*
Four samples of a hepatocyte suspension (7×10^6 cells/ml) were made 0.062nM (□), 23nM (○) or 70nM (△) with respect to ^{125}I-invertase. (*a*) Cell-associated ligand; (*b*) total uptake [cell-associated plus specifically degraded (increase in phosphotungstic acid-soluble) ligand at each time point].

parenchymal (Fig. 4) and non-parenchymal cells (Fig. 1) were comparable in magnitude. This shows that contamination of non-parenchymal cells in the parenchymal-cell preparation cannot account for the observed endocytosis of invertase in hepatocytes, because the contamination is less than 2% by cell number, which implies that the fraction of ligand molecules which went into non-parenchymal cells in Fig. 4 was also less than 2% of the total uptake.

Uptake of yeast invertase by parenchymal cells was decreased to 22% of the control value by 100mM-*N*-acetylglucosamine. The uptake was blocked by α-methyl mannoside (100mM) or by the Ca^{2+} chelator EGTA (3mM); the mannose-binding protein of hepatocytes is Ca^{2+}-dependent (Maynard & Baenziger, 1982; Mori *et al.*, 1983). The uptake was also strongly inhibited by mannan from *S. cerevisiae* (70% inhibition at 10μg/ml), confirming that mannose residues are essential to the uptake. Addition of a large excess of either asialo-fetuin or asialo-orosomucoid to the hepatocyte suspension did not influence the uptake of invertase. Neuraminidase treatment of the hepatocytes (30munits/ml for 10min at 37°C at pH7.4) had no effect on the uptake of yeast invertase; as expected (Tolleshaug & Berg, 1980), the enzyme sharply decreased the uptake of asialo-fetuin. These experiments confirm that the asialoglycoprotein receptor is not involved in the endocytosis of invertase.

We measured the uptake into hepatocytes of a small glycoprotein which contains one high-mannose glycan per molecule, namely RNAase B (M_r 13000), by a protocol closely similar to that of Fig. 2. The highest increase in cell-associated radioactivity was 1.5% of the total during 30min of incubation. We regard this as a negative result.

Binding of invertase to hepatocytes at 4°C

The rate of uptake of invertase and the amount of cell-associated invertase in hepatocytes were much lower than the corresponding values for asialo-glycoproteins in hepatocytes (Tolleshaug *et al.*, 1977; Tolleshaug, 1981). To obtain an indication of whether the difference is due to a lower binding capacity of the cell surface or a lower affinity of the receptor, binding of ^{125}I-invertase to hepatocytes at 4°C was studied.

At the lowest initial concentration (0.1nM), only 1.4% of the added ligand became cell-associated. The Scatchard (1949) plot of the binding data was strongly concave, indicating heterogeneity of binding sites (Fig. 5). The number of specific binding

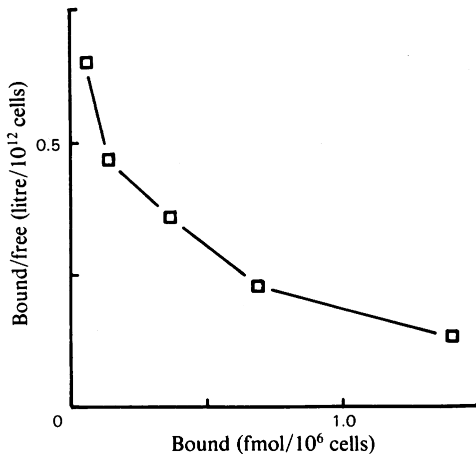


Fig. 5. *Binding of invertase to suspended hepatocytes at 4°C*

Samples of a cell suspension (10×10^6 cells/ml) were made 0.1–10nM in ^{125}I-invertase, which was diluted with unlabelled ligand as required. Immediately after the addition, zero-time samples were taken. There was no difference in the amounts of cell-associated ligand after 30 or 60min, indicating that equilibrium had been reached. From all measurements, 0.8% non-specific binding was subtracted. Each point is the mean of duplicate samples. The results are plotted according to Scatchard (1949).

sites could be estimated as roughly 10^3 per cell, and the average association constant was of the order of $10^8 M^{-1}$. This type of measurement at 4°C may not be relevant for the situation at physiological temperatures, as the receptors may be arranged differently in the plasma membrane. At 37°C, the amount of surface-bound invertase that is released by EGTA from the cell surface is too small to be measurable; on the other hand, the amount of surface-bound asialo-orosomucoid may be determined accurately (Tolleshaug, 1981; Tolleshaug *et al.*, 1982).

Intracellular distribution of invertase

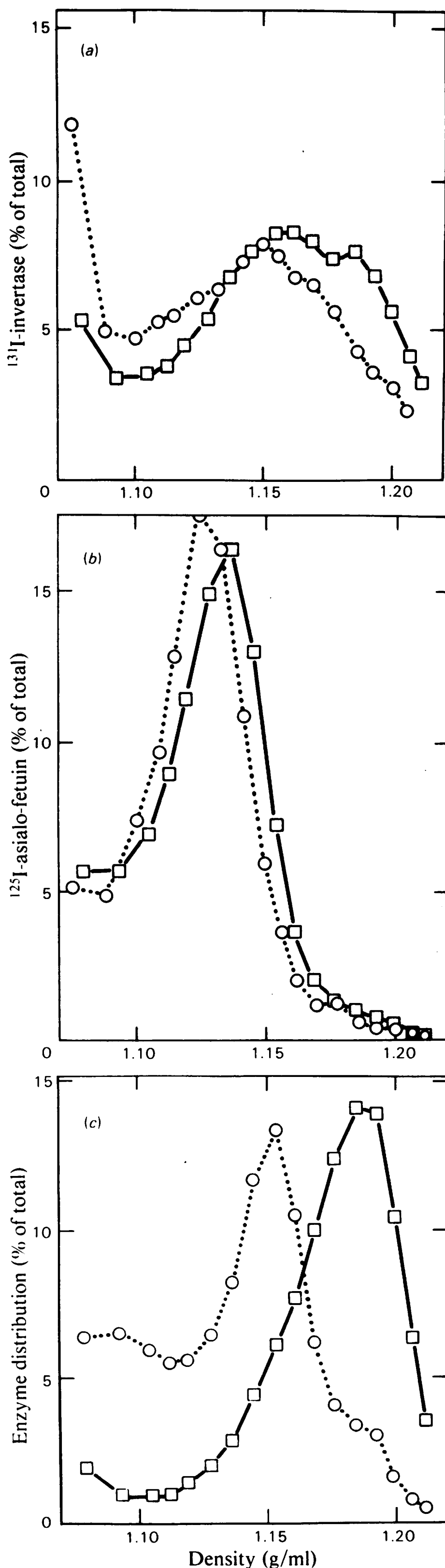
In order to study the intracellular transport and degradation of invertase, we fractionated hepatocytes by isopycnic centrifugation in sucrose gradients. As we wanted to compare the invertase system with the asialo-glycoprotein system, the cells were allowed to take up ^{131}I-invertase as well as ^{125}I-asialofetuin before fractionation.

The intracellular distribution of yeast invertase was different from that of asialo-fetuin, a glycoprotein ligand which is taken up by a different carbohydrate-specific receptor. Nearly all of the asialo-fetuin was recovered in a single tall peak; the density of the peak fraction increased from 1.13 to 1.14g/ml on incubation of the cells for 10min in the absence of ligand after the 10min uptake period (Fig. 6*b*). This shift towards higher density is real and reproducible; it presumably reflects processing of intracellular vesicles (Tolleshaug *et al.*, 1979; Tolleshaug & Berg, 1981). Over 90% of the cell-associated ^{125}I-asialo-fetuin entered the gradient.

Some 70% of the ^{131}I-labelled invertase entered the gradient from a homogenate of hepatocytes that had taken up invertase for 10min (over 80% after the cells had been incubated for an additional 10min at 37°C) (see the legend to Fig. 6). The distribution of invertase was broader than that of asialo-fetuin. After 10min of incubation of the cells with invertase, there was a single peak at 1.15g/ml (Fig. 6*a*). This is a significantly higher density than the peak of the asialo-fetuin distribution; 1.15g/ml is also the density of the peak of the plasma-membrane marker enzyme 5′-nucleotidase (Fig. 6*c*). The distribution of invertase extended into the same region of the gradient as the lysosomal marker enzyme (Fig. 6*c*), which was not the case with asialo-fetuin (Fig. 6*b*) from the same cells.

After washing of the cells and incubation for a further 10min at 37°C, an additional peak of ^{131}I-invertase was found at 1.19g/ml, which is the same as for the peak of hexosaminidase, the lysosomal marker enzyme. Roughly one-quarter of the ligand molecules in the gradient was associated with this peak. (A negligible amount of asialo-fetuin was recovered in this region of the gradient.) The difference in distribution of invertase after 10min of additional incubation shows that intracellular transport was taking place. Re-incubation of the cells for even longer periods revealed that, after 30min, all of the intracellular invertase was recovered close to the lysosomal marker enzyme at 1.18–1.19g/ml (Fig. 7). The rate of transfer of invertase into this region of the gradient was much faster than that of asialo-fetuin (Tolleshaug & Berg, 1981).

The total rate of degradation of cell-associated ligand may be studied by first allowing the cells to take up the ligand, then changing the medium in order to stop further uptake. In this type of experiment, intracellular transport as well as lysosomal proteolysis influence the rate of degradation that is measured (but the rate of uptake has no influence). As regards asialo-fetuin, the kinetics of degradation of cell-associated ligand is first-order, 0.9% of the intracellular molecules being degraded/min (Tolleshaug *et al.*, 1980). In similar experiments with ^{125}I-invertase, the rate constant was 0.4%/min. The reason for the low rate of proteolysis of invertase (relative to asialo-fetuin) may be related to the observation that, in some instances, attack on the peptide chain by lysosomal protein-



ases commences only after the carbohydrate has been removed (Winkler & Segal, 1984).

Another explanation for the low rate of proteolysis of invertase is a low rate of entry of this protein into the lysosomes. An indication of the validity of this argument may be obtained by examining the distribution profiles after subcellular fractionation. A large fraction of the intracellular invertase co-migrates with the lysosomes. If the increase in lysosome-co-migrating invertase indicates the rate of entry into lysosomes, then invertase enters at least as fast as asialo-fetuin. Thus the low rate of proteolysis of invertase is best explained by protection of the peptide portion by the large high-mannose chains.

Discussion

Previous work with isolated hepatocytes and mixtures of non-parenchymal cells indicated that all of the endocytic activity for mannose- or *N*-acetylglucosamine-terminated ligands resides with the non-parenchymal cells (Steer & Clarenburg, 1979). After injection into intact rats and determination of the distribution of labelled ligands by electron autoradiography, it was found that endothelial cells were much more active than Kupffer cells on a volume basis (Hubbard *et al.*, 1979). Conceivably, this distribution of the glycoproteins might reflect the flow of blood around the cells of the sinusoids.

This study confirms that isolated endothelial cells are very active in endocytosing mannose-terminated glycoproteins, e.g. RNAase B or invertase. Isolated endothelial cells or suspensions of mixed non-parenchymal cells endocytose the small

Fig. 6. *Intracellular distributions of ^{131}I-invertase and ^{125}I-asialo-fetuin in suspended hepatocytes*

Hepatocyte suspension (10ml; 7×10^6 cells/ml) was made 0.2nM with respect to ^{131}I-invertase and 0.04nM with respect to ^{125}I-asialo-fetuin. The incubation was continued at 37°C for 10min. Then 5ml of the suspension was put on ice; the rest of the cells were washed twice in ice-cold medium to remove extracellular ligands, and then post-incubated for 10min more at 37°C. Both samples were washed in ice-cold 0.25M-sucrose, homogenized and fractionated in sucrose gradients as described in the Experimental section. (*a*) Distributions of ^{131}I-invertase after 10min at 37°C (○) and after 10min plus 10min post-incubation in the absence of extracellular ligand (□). (*b*) Distributions of ^{125}I-asialo-fetuin after 10min at 37°C (○) or 10+10min (□) (as in *a*). (*c*) Distributions of marker enzymes for the plasma membrane (5′-nucleotidase, ○) and lysosomes (hexosaminidase, □). These distributions were identical in the 10min or 10+10min samples.

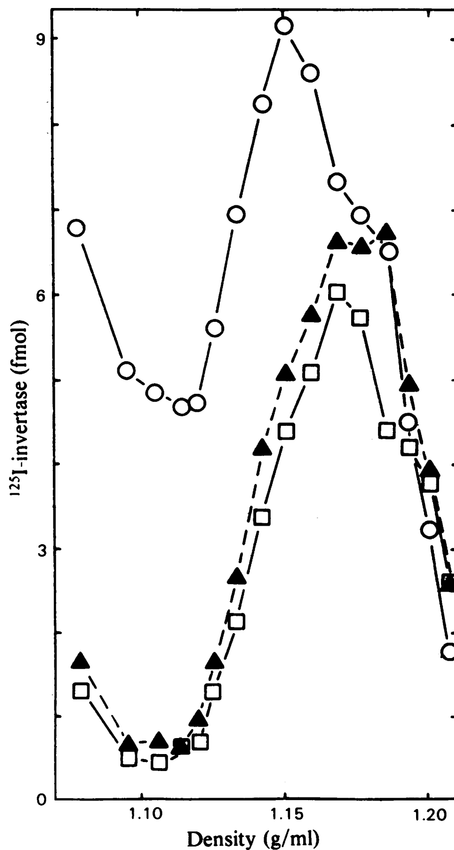


Fig. 7. *Intracellular transport of invertase as shown by subcellular fractionation*

Hepatocyte suspension (20ml) was made 0.7nM with respect to ^{125}I-invertase. The fractionation procedure was similar to that described in the legend to Fig. 6. Samples of the cell suspension were fractionated after 10min of incubation in the presence of ligand (○), and after 30 (▲) and 60 (□) min of post-incubation in the absence of extracellular ligand. The positions of the marker enzymes for the plasma membrane and lysosomes were the same as in Fig. 6(*c*).

ligand RNAase B as fast as they did invertase, which has 20 times higher M_r and many more mannose residues than RNAase B.

Up to now, endocytosis of mannose-terminated glycoproteins by hepatocytes has not been reported, even though the presence of a mannose-specific receptor in the cells has been confirmed. The specificity of the hepatocyte mannose receptor is different from that of the corresponding receptor on Kupffer and endothelial cells (Maynard & Baenziger, 1982; Mori *et al.*, 1983). The two mannose-binding receptors may also be distinguished immunologically (Mori *et al.*, 1983). The total binding capacity of homogenized hepatocytes is approx. 60000 molecules of *N*-acetylglucosamine-terminated orosomucoid per cell (Maynard & Baenziger, 1982) or about 300000 molecules of yeast mannan per cell (Mori *et al.*, 1983). In both of these studies, it was found that hepatocytes contained more than 90% of the total binding capacity of the liver for these glycoproteins.

In the present study, hepatocytes were shown to take up and degrade yeast invertase. The uptake was saturable, Ca^{2+}-dependent, and could be inhibited by α-methyl mannoside, *N*-acetylglucosamine or yeast mannan. Asialo-fetuin or asialo-orosomucoid did not inhibit the uptake of yeast invertase, neither did neuraminidase treatment of the hepatocytes influence the uptake of invertase, showing that the asialo-glycoprotein receptor was not involved. Thus endocytosis of invertase in hepatocytes is mediated by a receptor which has similar binding properties to the mannose/*N*-acetylglucosamine-binding protein, which has been identified in extracts from hepatocytes.

In contrast with yeast invertase, RNAase B was taken up to a negligible extent in hepatocytes. The reason for the difference in uptake rates between these two ligands is probably not a low affinity of the isolated receptor for RNAase B (Maynard & Baenziger, 1982). The affinity of the receptor on the cell surface could be different from that of the isolated receptor, perhaps to the extent that binding of the ligand to several receptor molecules is required. Invertase, being a large multivalent ligand, would allow this. A different explanation is that if there are mannose-specific receptors on the hepatocyte cell surface, they might be occupied by high-mannose glycans of membrane glycoproteins. Analogously, most of the asialo-glycoprotein receptors may become occupied if the galactose residues of membrane glycoproteins are exposed after treatment of the cell surface with neuraminidase (Tolleshaug & Berg, 1980). The local concentration of glycoprotein ligands in the membrane may be assumed to be high under these conditions. Consequently, ligands in the medium may be expected to compete only if they bind with very high affinity, a condition which is met more easily by multivalent ligands.

Uptake of invertase into hepatocytes is mediated by a relatively small number of sites with high (but variable) affinity. The reason for the variable affinity constants may be that each invertase molecule binds to different numbers of mannose-binding receptors on the cell surface. The specific binding capacity of the hepatocyte for invertase is less than 2% of the binding capacity for asialo-glycoproteins (Schwartz *et al.*, 1980; Tolleshaug & Berg, 1979), whereas the respective association constants are of the same order of magnitude. The possibility remains that the rate of internalization

of surface-bound invertase is different from the rate of internalization of asialo-glycoproteins.

Comparison of the surface binding capacity as estimated by us with the total binding capacity of homogenized hepatocytes from previous studies (Maynard & Baenziger, 1982; Mori *et al.*, 1983) shows that 90% or more of the binding capacity is intracellular. This distribution is in rough agreement with the distribution of asialo-glycoprotein-binding activity between the interior and the exterior of the cell (Pricer & Ashwell, 1976; Blomhoff *et al.*, 1982). The surface binding capacity of hepatocytes for asialo-glycoproteins has been shown to vary widely between different determinations (Schwartz *et al.*, 1980). 'Leakage' of the intracellular mannose receptor on to the cell surface may vary according to the methods of cell preparation etc. Contamination of serum or other proteins in the culture medium by mannose-containing glycoproteins may be responsible for lowering of the apparent cell-surface binding capacity. For these reasons, we are not surprised that variable results have been obtained in experiments on the uptake of mannose-terminated glycoproteins by hepatocytes.

At 10min after the addition of ligand to the hepatocytes, most of the invertase was closely associated with the plasma-membrane marker 5'-nucleotidase, as shown by fractionating a sample of the cell suspension in a sucrose gradient. On fractionation of cells after additional incubation in the absence of ligand, an effect of intracellular transport was evident: a significant portion of the intracellular invertase had become associated with the lysosomes. In both cases, the subcellular distribution of invertase was different from that of asialo-fetuin, and it was also obvious that prolonged incubation changed the intracellular distribution of invertase in a different manner from the change in asialo-fetuin distribution. Thus the route of intracellular transport of invertase is not identical with that of asialo-fetuin.

In the degradation of asialo-fetuin by hepatocytes, the rate-limiting step is transport of the ligand into the lysosomes (Tolleshaug *et al.*, 1979; Tolleshaug & Berg, 1981). The ^{125}I-asialo-fetuin that reaches the lysosomes is quickly degraded; no intact ligand is recovered in these organelles (a small amount is observed after the cells have been incubated for about 2h). Invertase does accumulate in the lysosomes after only 20min of incubation; the reason for this difference from asialo-fetuin may be that invertase enters the lysosomes much faster, so that lysosomal proteolysis becomes the rate-limiting step. The susceptibility of invertase to proteolysis by lysosomal enzymes may also be lower than that of asialo-fetuin, so that invertase accumulates in the lysosomes even though it is transferred into them at the same rate as asialo-fetuin. Both factors may operate to produce the observed accumulation of invertase.

When ^{131}I-labelled invertase was injected into a rat, and the liver cells were separated, a little less than half of the radioactivity in the liver was recovered in the hepatocytes, indicating that the mannose-specific receptor of hepatocytes is active in endocytosis of high-mannose proteins *in vivo*. Its function remains a subject for conjecture, but it is an attractive possibility that the receptor binds lysosomal enzymes, as suggested by Sly (1982) and Neufeld & McKusick (1983). A mannose-specific receptor on hepatocytes was found to be responsible for a small fraction of the uptake of α-*N*-acetylglucosaminidase into these cells (Ullrich *et al.*, 1979).

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References

Berg, T. & Boman, D. (1973) *Biochim. Biophys. Acta* **321**, 585–596

Berg, T. & Tolleshaug, H. (1980) *Biochem. Pharmacol.* **29**, 917–925

Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520

Blomhoff, R., Tolleshaug, H. & Berg, T. (1982) *J. Biol. Chem.* **257**, 7456–7459

Blomhoff, R., Holte, K., Naess, L. & Berg, T. (1984) *Exp. Cell Res.* **150**, 186–193

Granum, P. E. & Skjelkvaale, R. (1977) *Acta Pathol. Microbiol. Scand. Sect. B* **85**, 89–94

Hubbard, A. L., Wilson, G., Ashwell, G. & Stukenbrok, H. (1979) *J. Cell Biol.* **83**, 47–64

Lehle, L. (1980) *Eur. J. Biochem.* **109**, 589–601

Maynard, Y. & Baenziger, J. U. (1981) *J. Biol. Chem.* **256**, 8063–8068.

Maynard, Y. & Baenziger, J. U. (1982) *J. Biol. Chem.* **257**, 3788–3794

Mori, K., Kawasaki, T. & Yamashina, I. (1983) *Arch. Biochem. Biophys.* **222**, 542–552

Neufeld, E. F. & McKusick, V. (1983) in *The Metabolic Basis of Inherited Disease*, 5th edn. (Stanbury, J. B., Wyngaarden, J. B., Frederickson, D. S., Goldstein, J. L. & Brown, M. S., eds.), pp. 778–787, McGraw-Hill, New York

Nilsson, M. & Berg, T. (1977) *Biochim. Biophys. Acta* **497**, 171–182

Pricer, W. E. & Ashwell, G. (1976) *J. Biol. Chem.* **251**, 7539–7544

Prieels, J. P., Deschuyteneer, M., May, C. & Wanson, J. C. (1979) in *Glycoconjugates* (Schauer, R., Boer, P., Buddecke, E., Kramer, M. F., Vliegenthart, J. F. G. & Wiegandt, H., eds.), pp. 502–503, Georg Thieme Publishers, Stuttgart

Redshaw, M. R. & Lynch, S. S. (1974) *J. Endocrinol.* **60**, 527–528

Sakaguchi, G., Uemura, T. & Riemann, H. (1973) *Appl. Microbiol.* **27**, 762–767

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672

Schwartz, A. L., Rup, D. & Lodish, H. F. (1980) *J. Biol. Chem.* **255**, 9033–9036

Seglen, P. O. (1976) *Methods Cell Biol.* **10**, 29–83

Sly, W. S. (1982) in *The Glycoconjugates* (Horowitz, M. I., ed.), vol. 4, part B, pp. 3–25, Academic Press, New York

Stahl, P. D., Rodman, J. S., Miller, M. J. & Schlesinger, P. H. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1399–1403

Stahl, P., Schlesinger, P. H., Sigardson, E., Rodman, J. S. & Lee, Y. C. (1980) *Cell* **19**, 207–215

Steer, C. J. & Clarenburg, R. (1979) *J. Biol. Chem.* **254**, 4457–4461

Tolleshaug, H. (1981) *Int. J. Biochem.* **13**, 45–51

Tolleshaug, H. & Berg, T. (1979) *Biochem. Pharmacol.* **28**, 2919–2922

Tolleshaug, H. & Berg, T. (1980) *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1155–1164

Tolleshaug, H. & Berg, T. (1981) *Exp. Cell Res.* **134**, 207–217

Tolleshaug, H., Berg, T., Nilsson, M. & Norum, K. R. (1977) *Biochim. Biophys. Acta* **499**, 73–84

Tolleshaug, H., Berg, T., Frolich, W. & Norum, K. R. (1979) *Biochim. Biophys. Acta* **585**, 71–84

Tolleshaug, H., Berg, T. & Holte, K. (1980) *Eur. J. Cell Biol.* **23**, 104–109

Tolleshaug, H., Berg, T. & Holte, K. (1982) *Biochim. Biophys. Acta* **714**, 114–121

Trimble, R. B. & Maley, F. (1977) *J. Biol. Chem.* **252**, 4409–4412

Ullrich, K., Basner, R., Gieselmann, V. & von Figura, K. (1979) *Biochem. J.* **180**, 413–419

Warr, G. A. (1980) *Biochem. Biophys. Res. Commun.* **93**, 737–745

Winkler, J. R. & Segal, H. (1984) *J. Biol. Chem.* **259**, 1958–1962