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Uptake and endocytic pathway of transferrin and iron in perfused rat liver

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Uptake and distribution of transferrin and iron in perfused rat liver are dependent on perfusion temperature, time and uptake affinity. Transferrin passes at least two different compartments on its receptor-mediated recycling pathway, which are separable by centrifugation in a shallow Nycodenz gradient. Perfusion at lowered temperature (16 °C) is sufficient for internalization of transferrin and iron. Passage of radiolabelled iron to other than endosomal compartments as well as recycling of labelled transferrin are largely suppressed at this perfusion temperature, as much less is released by further perfusion with unlabelled transferrin than at 4 °C where the ligand is largely washed off the surface, or 37 °C, where the recycling pathway is operating. But also at lowered temperature only a part of the iron in endosomal fractions can be assigned to transferrin. A considerable part of the total uptake of transferrin and iron can be attributed to low-affinity mechanisms even at very low transferrin concentrations. Transferrin receptors are concentrated in endosomal fractions in comparison to fractions representing different plasma membrane domains of the liver. Endosomal fractions specifically display detergent-activated NADH–acceptor oxidoreductase which may be part of the iron uptake system.

Introduction

The liver is of prime importance in regulating the iron metabolism of mammals. It is the major iron storage organ and the site of biosynthesis for the iron-transporting plasma glycoprotein transferrin, which is itself regulated by iron [1], and it steadily takes up 10–20% of the circulating iron, a part of this stemming from transferrin [2,3,4].

In a number of cultured cell types as well as in erythroid precursor cells, in placenta and in other tissues the pathway of iron uptake via receptor-mediated endocytosis of transferrin is well established (see for example, Refs. 5–11). It is beyond the scope of this paper to give even an approximate literature overview. In the case of the liver the endocytic uptake mechanism has been questionned on the grounds of its dependence on oxygen concentration and on redox reactions [12–14], and a direct transfer of iron at the plasma membrane is seen as a possible alternative.

On the other hand, studies on the subcellular distribution after injection of labelled transferrin or liver perfusion have provided strong evidence for receptor-mediated endocytosis and recycling of transferrin in liver [15–18].

Using temperature-controlled perfusion of the isolated rat liver [19] we demonstrate here that transferrin passes through at least two endocytic compartments on its recycling pathway, which are separable by their buoyant density [20], and that iron release starts very early after internalisation, as described for other endocytic systems [21,22]. The subcellular endosomal fractions displaying high concentrations of transferrin receptors also show a detergent activable pyridine nucleotide oxidase which may be important for reduction of released iron to enable its facilitated transfer through the endosomal membrane.

Materials and Methods

Ligands. Human transferrin was purchased from Sigma and loaded with unlabelled or radioactive (^{59}Fe, obtained as $FeCl_3$ from ICN) iron according to Bates and Schlabach [23]. Unlabelled diferric transferrin was

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iodinated with Iodogen (Pierce) and $Na^{125}I$ (Behringwerke) according to Fraker and Speck [24]. The biological equivalence of the labelled transferrins was checked by measuring binding capacity and affinity in K562 cells [9]. A mixture of both was used for perfusion and uptake studies. Human transferrin is bound by rat hepatocyte transferrin receptors with the same or higher affinity as rat transferrin [25,26].

Asialoorosomucoid (ASOR), a gift from Dr. R. Fuchs (Institute of Experimental Pathology, University of Vienna), was iodinated in the same way.

Liver perfusion. The protocol of Dunn and Hubbard [19] was followed for temperature controlled perfusion of the isolated rat liver (liver weights 12 fo 14 g). The perfusion fluids were gassed with O_2/CO_2 (95%/5%), the flow rate was 30 ml/min. The original perfusion temperature was registered with a thermometer directly at the buffer entrance into the organ. Perfusion with radioactive ligand was terminated with ice-cold perfusion buffer. The temperature of the liver fell to below 8°C within less than one minute.

Perfusion with transferrin was carried out in a recirculating system with 60 ml of perfusion fluid to load the recycling pathway of the liver. The concentration of diferrric transferrin (100 nM) was high enough to keep it practically constant (> 80% of the original amount) throughout the perfusion time. To study the release of iron and transferrin the perfusion with radioactive ligand was terminated with a 2-min flush with cold buffer, and then new buffer containing 120 nM unlabelled diferric transferrin was perfused through the liver for 20 min at a rate of 30 ml/min at the indicated temperature and collected for determination of radioactivity by liquid scintillation counting in a Packard counter. The radioactivity of iodine and iron was discriminated by an appropriate spectral analysis by the counter software. Further perfusion did not enhance release of ligand. When the liver was further perfused with icecold buffer without unlabelled ligand, no release of radioactivity occurred.

Perfusion with ASOR was carried out in the following way. The liver was perfused with ASOR-containing buffer (10 μg/ml) for 1 min at the desired temperature, then the perfusion was switched to pure recirculating buffer at the same temperature and the perfusion continued for the required time. The total perfusion time includes the pulse-minute. In some experiments, this second buffer contained ^{59}Fe-labelled transferrin to check the comparability of the two procedures. This was always the case (see Results).

Liver fractionation. Subcellular fractionation of livers was carried out with three livers for one experiment according to Evans and Flint [20] with some modifications: All solutions of sucrose and Nycodenz were buffered with 5 mM Tes (pH 7.4). Sucrose gradient fractionation was carried out in a Z-60 zonal rotor in a Beckman L8-55 centrifuge at 55000 rpm for 3 h. 100 ml of sample were layered onto 150 ml of gradient (15–35% w/w) and 45 ml of 37% (w/w) sucrose resting on a cushion of 55% (w/w) sucrose. Fractions of 10 ml were collected, and aliquots taken for determination of radioactivity. The results are displayed as frequency histograms. The separation of endocytosed transferrin from marker enzymes for plasma membranes, Golgi apparatus and endoplasmic reticulum has been shown before [18].

Fractions from two regions of the sucrose gradient assigned to 'early' (density 1.12–1.14 g/ml) and 'late' (density 1.095–1.12 g/ml) endosomes were pooled separately, diluted to below 15% (w/w) sucrose, layered on Nycodenz gradients ranging from 13.5 to 27% (w/v) resting on 55 % (w/w) sucrose, spun overnight at 21000 rpm in the SW27 rotor and fractionated separately into 17 fractions per gradient. Nycodenz was obtained from Dr. Molter Company, Heidelberg.

The corresponding figures show the percentage of total radioactivity of the Nycodenz gradient after perfusion for 10 min at 37°C in a fraction of particular density plotted against refractive index as a measure of density. The radioactivity of all fractions of equal density was added to yield total radioactivity in the given density interval. The area below each individual curve is a measure of the relative amount of radioactivity found in the Nycodenz gradient under the particular perfusion conditions. Fractions from the DN-1 (density 1.075–1.11 g/ml), DN-2 (density 1.117–1.13 g/ml) and DR-region (sucrose/Nycodenz border) of the Nycodenz gradients, corresponding to 'late', 'early' and 'receptor-recycling' endosomes [20] were pooled, diluted with water, centrifuged at 28000 rpm for 2 h in the 55.2 Ti rotor and resuspended with a small Dounce homogenizer in sucrose-Tes medium.

For receptor-binding assays, endosomes and plasma membrane fractions from untreated livers were isolated according to the procedure described above and according to Wisher and Evans [27] with the modification of Evans et al. [28], respectively.

Immunoprecipitation. A goat anti-human transferrin antibody was purchased from Bio-Yeda and used for the precipitation of transferrrin. Endosomes were solubilized in phosphate buffered saline (150 mM NaCl, 10 mM phosphate (pH 7.4), PBS) containing 0.1% Triton X-100. The suspensions were incubated with antibody at 4°C overnight and the precipitates separated by centrifugation in a Microfuge. The pellet was washed with PBS and counted. Completeness and specifity of precipitation and transfer were controlled with pure transferrin and unspecific goat anti-rabbit IgG, respectively.

Soluble receptor assay. The procedure of Lamb et al. [8] was used to estimate the concentration of binding sites in subcellular fractions. Unspecific binding was

estimated in presence of a 100-fold surplus of unlabelled transferrin. Membranes were dissolved in 0.1% Triton X-100, 150 mM NaCl, 5 mM Tris (pH 8) and incubated at 4 °C for 2 h with 100 nM ^{125}I-transferrin. The transferrin-receptor complex was precipitated in the cold in the presence of 1 mg/ml IgG with 45% saturated ammonium sulfate. After one more hour on ice, the precipitate was pelleted by centrifugation, the supernatant was aspirated and the pellet counted in a gamma-counter.

Enzyme assays. NADH–ferricyanide oxidoreductase and NADH–cytochrome-*c* reductase (rotenone-insensitive) were assayed according to described procedures [29]. For assays in the presence of saponin, the samples were diluted with saponin-containing buffer (final concentration 0.02%) 10 min before the actual assay.

Results

The amount of transferrin and iron taken up by the perfused liver was dependent on the perfusion temperature. Perfusion at 4 °C for 20 min resulted in equal uptake of 0.5% of total radioactive transferrin protein and iron in the perfusion fluid. This percentage rose to slightly more than 1% at 16 °C. Perfusion for longer time did not enhance the amount of transferrin associated with the liver at this temperature, while the increase in iron uptake was also negligibly small (not shown).

At the physiological temperature of 37 °C the time dependence of uptake from the perfusate was determined. The amount of transferrin protein taken up increased from an average of 2.73% (2.1% for iron) of the total perfused radioactivty after 3 min to 4–5% after l0min which then remained constant. Uptake of iron increased with time to 11% after 20 min. Uptake of transferrin and iron after 20 min at 37 °C could be suppressed to approximately 0.8% of total perfused label by a 20-fold surplus of unlabelled transferrin, which was 20% of total uptake for transferrin and 4% for iron (Table I). Thus part of the observed uptake was due to low-affinity mechanisms even at the low concentrations used and despite the higher affinity of human compared to rat transferrin for the rat receptor [25,26].

Iron donation was apparently less efficient in this suppressible pathway.

When the perfusion was switched to buffer with the respective temperature containing 120 nM unlabelled transferrin a part of the radioactivity was released. Release of two thirds to three quarters of the transferrin protein and of the iron were seen after 20 min perfusion and 10 min reperfusion at 4 °C. At 16 °C with the same time protocol only around 30% of the iron and 45% of the transferrin were released, indicating internalization of most of the transferrin without efficient recycling. At 37 °C, again around 65% of the transferrin, but also 20 to 35% of the iron were released (Table I).

To study the pathway of transferrin and iron to different endosomal compartments after liver perfusion at different times and temperatures, the analysis of the postlysosomal supernatants by density gradient centrifugation was calibrated by the perfusion and internalization of ASOR whose intracellular pathway in liver cells is very well described [30–34]. The perfusion protocol was not the same for ASOR-perfusion as for transferrin. Because of the much higher capacity of liver for uptake of asialoglycoproteins compared to transferrin [35] and because most of the ASOR is not recycled [36], perfusion with the ligand itself was carried out for only 1 min followed by pure buffer for the indicated times. When this perfusion buffer contained diferric transferrin with radiolabelled iron, the same subcellular distribution of iron was found as in perfusion experiments with transferrin alone.

Carrying out the perfusion and uptake experiments with transferrin in a manner where the ligand is first bound to the surface at 4 °C and then allowed to enter the cells by temperature switch yielded very low

TABLE I

Uptake and release of transferrin and iron by the perfused rat liver

Mean ± standard deviation from 5–7 experiments or data from two experiments (perfusion at 4 °C and release) are shown. Release (right column) was estimated by perfusion of the liver with buffer containing 120 nM unlabelled transferrin for 10 min.

Perfusion temperature (°C)	Perfusion time (min)	Uptake (% of total perfused ligand)		Release (% of liver content)	
		transferrin	iron	transferrin	iron
4	20	0.5	0.5	66–68	74–75
16	20	1.34 ± 0.25	1.13 ± 0.32	41–45	27–35
37	3	2.73 ± 0.32	2.1 ± 0.56		
37	10	4.26 ± 0.4	5.06 ± 0.76		
37	20	4.52 ± 0.23	11.5 ± 1.13	62–72	20–35
37, + 2 μM unlabelled transferrin	20	0.85	0.8		

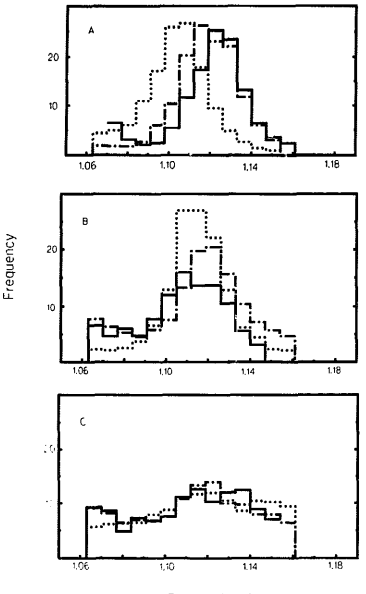

Fig. 1. Frequency histograms for distribution of asialoorosomucoid (A), transferrin (B) and iron (C) from a postlysosomal rat liver fraction in sucrose density gradients (15 to 35% (w/w)) after centrifugation for 3 h at 55000 rpm. ———, Perfusion temperature 37 °C, perfusion time 3 min; — — —, 37 °C, 10 min; ·–·–·, 16 °C, 20 min.

amounts of iron uptake, forfeiting the possibility of quantitiative analysis in the density gradients. Loading of the liver endocytotic system by recirculating ligand perfusion was a necessary prerequisite for the experiments described here.

The postlysosomal supernatants of differential centrifugation were subjected to sucrose density gradient centrifugation (Fig. 1). We have previously shown that a large part of the transferrin taken up by the liver is located in low density membrane fractions of microsomal size [18]. The fraction actually entering the sucrose gradient contained approximately half of the total liver-associated radioactivity of transferrin. After perfusion at 16 °C, all endocytosed labels peaked at a density around 1.11–1.13 g/ml, with iron also found at lower and higher densities. Perfusion at 37 °C brought about a shift of ASOR to lower density (1.09–1.12 g/ml) after 10 min, but did not lead to significant changes in the distribution of transferrin protein in sucrose gradients, while iron under these conditions showed an even flatter distribution throughout the gradient with a time-dependent rise of the label in the lighter (density 1.08–1.10 g/ml) and the heavier fractions (1.14–1.16 g/ml). The radioactivity was alway completely recovered in the gradient with respect to the starting fraction, which was also true for the Nycodenz gradients (see below).

Comparison of transferrin protein and iron content of the gradient fractions based on the specific radioactivities of the original ligands yielded a quantitative measure of the distribution difference between perfusions for 20 min at 16 °C and for 3 min or 10 min at

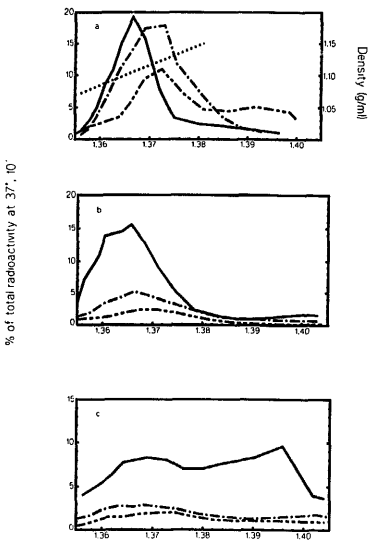

Fig. 2. Distribution of asialoorosomucoid (A), transferrin (B) and iron (C) from a low sucrose density gradient fraction (see Fig. 1) in isotonic Nycodenz density gradients (13.5 to 27% (w/v) resting on a cushion of 55% (w/w) sucrose) after overnicht centrifugation at 21000 rpm. ·–·–·, Perfusion temperature 37 °C, perfusion time 3 min; ———, 37 °C, 10 min; ·–·–·, 16 °C, 20 min. Percent of total radioactivity of the gradient after perfusion at 37 °C for 10 min found in particular fractions (16 to 17 each) is plotted. ·····, Density.

TABLE II

Ratio iron / transferrin protein in sucrose density gradient fractions, calculated from the specific acitivity of the ligands

Density (g/ml)	Perfusion temperature and time		
	16 °C 20 min	37 °C, 3 min	37 °C, 10 min
1.06	1.24	1.0	6.0
1.07	1.6	0.75	6.95
1.076	2.0	1.02	6.0
1.083	1.95	1.3	4.2
1.09	2.2	1.12	2.9
1.097	2.3	0.84	1.62
1.105	0.9	0.68	0.99
1.112	0.92	0.57	0.96
1.119	0.73	0.41	1.3
1.126	1.35	0.38	1.77
1.133	1.12	0.56	3.3
1.14	1.2	0.56	3.3
1.147	2.3	1.16	10.7
1.157	4.0	1.18	10.5

37 °C (Table II). Separation of iron and transferrin was not completely blocked at 16 °C. The presumed location of the low density endocytic vesicles in the gradient corresponds to the lowest iron/protein ratio. This ratio minimum shifted from a density around 1.12 after 3 min at 37 °C (the peak density also seen at 16 °C) to one around 1.112 g/ml on longer perfusion time. This shift is invisible when considering the frequencies of radioactivity alone.

Sucrose density fractions representing the early (density 1.12–1.14 g/ml, corresponding to the peak of ASOR at 16 °C) and late (density 1.095–1.118 g/ml, corresponding to the ASOR-maximum at 37 °C, 10 min) endosomal regions [20] were pooled separately and subjected to fractionation in Nycodenz gradients. This fractionation yielded visible differences due to perfusion time and temperature for distribution of transferrin, too (Fig. 2). Results presented in this figure are shown in relation to the total radioactivity in the gradient after perfusion for 10 min at 37 °C. Thus the area below the curves is a measure of the relative total label found under different conditions. ASOR was pulse-chased (see Methods), therefore the amount found at 37 °C was independent of perfusion time. Perfusion at 16 °C directed all labels to peaks in a density region typical for 'early endosomes' (DN-2, 1.117–1.13 g/ml) reached after 1–3 min at 37 °C [20,37,38].

The distribution of the labelled protein ligands following perfusion at 37 °C changed with perfusion time: After 10 min there was a pronounced shift of the ligands to lower densities (1.075–1.11 g/ml) of the gradient corresponding to the DN-1 or 'late' endosome region, compared to the 16 °C or the short time 37 °C perfusion.

Iron from the 'endosomal' region of the sucrose gradient was partly found at the low border of the Nycodenz gradient, this part rising with perfusion temperature and time.

Pooling and centrifugal concentration of the high- and low-density gradient fractions to obtain 'early' (DN-2) and 'late' (DN-1) endosomes, as well as a Nycodenz/sucrose-border fraction (DR) [20] allowed a comparison of their respective transferrin and iron content (Table III). There was little change in the Fe/protein ratio in the DR-fraction, though the total content was much lower after a 16 °C perfusion (see Fig. 2), whereas at this low temperature there was a marked increase of the iron/transferrin ratio in the other two fractions. In all cases, only part of this iron was immunoprecipitable by an anti-transferrin antibody which completely immunoprecipitated transferrin itself. The percentage of immunoprecipitable iron relative to the iron/transferrin ratio was independent of the perfusion temperature in the two endosomal fractions, but was much higher in the DR-fraction after low-temperature perfusion, whereas no immunoprecipitable iron was found in this fraction after perfusion at 37 °C (Table III). Iron in the DR-fraction after the low perfusion probably stemmed from heavy endosomes overlapping in density with the bottom of the Nycodenz gradient. Thus iron release from transferrin was slowed down appreciably at 16 °C, as was endocytosis, but was not completely blocked, and neither was further passage of iron, as also indicated by its appearance in sucrose low and high density gradient fractions (Fig. 1, Table II).

Total transferrin receptors in subcellular membrane fractions were compared by a soluble receptor ligand-binding assay [8]. Based on their protein content, endosomal fractions had a much higher concentration of receptors than plasma membranes, even those derived from the sinusoidal membrane [27] (Table IV). Endosomal fraction DN-1 and DN-2, but not DR, displayed

TABLE III

Ratio iron / transferrin and immunoprecipitation of iron with anti-transferrin antibody in endosomal subfractions DN-1, DN-2 and DR

Data are from one typical experiment.

Fraction	Perfusion temperature	Fe/protein	% of iron immunoprecipitated
DN-1	37 °C	0.16	7.6
DN-2		0.20	7.3
DR		0.86	0
DN-1	16 °C	0.56	22.5
DN-2		0.71	29
DR		0.96	38

TABLE IV

Soluble transferrin binding site assay in rat liver subcellular membrane fractions

NL, 'nuclear light plasma membranes' (bile canalicular membranes), NHa, 'nuclear heavy plasma membranes a' (lateral plasma membrane), NHb, 'nuclear heavy plasma membranes b' (contiguous membranes); ML, 'microsomal light plasma membranes' (sinusoidal membranes); MR, microsomal residual membranes (mixture of various microsomal membranes). Unspecific binding in presence of a 100-fold surplus of unlabelled transferrin is subtracted.

Fraction	Transferrin binding sites/mg of membrane protein ($\times 10^{-11}$) (mean ± S.D. from three experiments)
DN-1	18.6 ±9.0
DN-2	6.9 ±1.13
DR	0.18±0.10
NL	0
NHa	0.16±0.08
NHb	0
ML	0.52±0.15
MR	0.35±0.12

stimulation of NADH–ferricyanide reductase activity by low concentrations (0.02%) of saponin sufficient to break vesicle latency. This detergent activiation was not detected in a microsomal fraction obtained by dilution and centrifugation for 1 h at 100000 × *g* of a heavy sucrose density gradient fraction where 90% of the total activity of NADPH–cytochrome-*c* reductase was found [18].

NADH–cytochrome-*c* reductase which was also detected in endosomal fractions was inhibited by the detergent (Table V).

Discussion

The pathway of iron into the liver and especially into its parenchymal cells is still a matter of debate. Most published evidence [15–18] suggests receptor-mediated endocytosis and recycling of transferrin through an acidic endocytic compartment of hepatocytes, where iron is released while the protein remains bound to its receptor throughout the recycling process. Other models have, however, been discussed for the liver [12–14]. In any case, there is considerable uptake of transferrin iron by low-affinity mechanisms [39]. Temperature-dependent uptake into and release from liver presented here argues in favour of a specific endocytotic mechanism. Release of transferrin on reperfusion with a surplus of unlabelled ligand at the original perfusion temperature was more efficient at 4 °C and at 37 °C than at 16 °C. For iron, the sequence was 4 °C > 16 °C > 37 °C. If transferrin were bound to the surface membrane only, one would not expect a releasing efficiency minimum at 16 °C, where membrane fusion events and therefore recycling is inhibited [40,41].

A surplus of unlabelled transferrin in the perfusion buffer suppressed uptake, but a significant part of transferrin (20%) was still taken up by the liver. Comparison with iron uptake under the same conditions indicated incomplete iron release from this part of transferrin. Subcellular fractionation of the liver after such a perfusion experiment (20 min at 37 °C with a surplus of unlabelled transferrin, not shown) indicated that approx. 60% of the protein label was located in the cytosolic fraction, probably representing trapped perfusion fluid. Exact kinetic studies would have to be done to confirm this observation.

According to the temperature- and time-dependent differences in subcellular distribution of transferrin and iron in Nycodenz gradients (Fig. 2), transferrin passes at least two different compartments corresponding in density to 'early' (DN-2) and 'late' (DN-1) endosomes [20]. There is no evidence for a separate receptor recycling compartment coincident with that of the asialoglycoprotein receptor (DR), because this fraction contained very few transferrin receptors and did not carry appreciable amounts of recycling transferrin. According to the distribution seen here the recycling compartment of transferrin is coincident with late endosomes in its density in Nycodenz, as seen from comparison with ASOR.

Transferrin, transferrin receptors, asialotransferrin and ASOR have all been immunolocalized together with an antibody to the asialoglycoprotein-receptor in the same population of low density vesicles (density in sucrose around 1.10 g/ml) from liver 10 to 20 min after intravenous injection of the respective ligand [15]. 14 min after onset of uptake of ASOR at 37 °C 75% of the ligand were found associated with the receptor in a fraction corresponding to 'early endosomes' in sucrose. The rest had passed to a vesicle population of lower density free of receptor [34], which can fuse with lysosomes.

TABLE V

NADH–ferricyanide reductase and NADH–cytochrome-c reductase activities in endosomal and microsomal fractions and change by 0.02% saponin

Mean ± S.D. from three experiments. n.s., no significant change.

Fraction	Acceptor	Specific activity (μmol/min per mg protein)	% change by detergent
DN-1	Ferricyanide	1.42±0.035	+87±27
DN-2		2.35±0.21	+17± 4.5
DR		1.55±0.21	n.s.
Microsomes		5.7 ±0.4	n.s.
DN-1	Cytochrome *c*	0.98±0.035	−17± 5.7
DN-2		1.25±0.071	−38±10.6
DR		2.35±0.07	−34± 7
Microsomes		4.1 ±0.2	n.s.

In our hands the bulk of ASOR had moved to a lower density compartment after a 10 min perfusion at 37 °C, a shift visible in sucrose as well as in Nycodenz (Figs. 1 and 2). In our case, microsomes were not collected by centrifugation and floated in the gradient [34], but were layered on top of the sucrose gradient as postlysosomal supernatant. The different treatment may result in different buoyant densities in sucrose. Transferrin-containing vesicles cannot be expected to be receptor-negative nor to fuse with lysosomes, but have been shown to fuse with other vesicles on their recycling pathway [42] and meet with the secretory branch of the membrane recycling mechanism [43], which means that transferrin passes more than one compartment of the endocytotic space.

A low temperature (16 °C) recycling of transferrin was inhibited and the protein ligand was largely confined to the early endocytic compartment.

The DN-1- or 'late' endosome fraction has a relatively high concentration of transferrin receptors. It may represent a mixture of 'recepter positive' and 'receptor negative' late multivesicular (and thus light) endocytic vesicles.

The terms 'early' and 'late endosomes' seem to be too simple to reflect the complexity of the endocytic pathway in the liver. Release of iron from transferrin was still partly active at 16 °C. This can be judged from the localization of iron in the density gradients which does not completely coincide with that of transferrin, and from immunoprecipitation of iron by anti-transferrin, which was incomplete under all conditions. As indicated by studies in other systems, iron release does not need a very low pH [44], but further passage of the metal may be blocked by insufficient acidification [21]. Free ferric ions are practically non-existent even under the slightly acidic conditions found in endosomes and must be bound either to a low molecular weight chelator or to an endosomal membrane protein [45].

There is considerable evidence that further processing of iron involves reduction [13,46–50], but the reduction step has not been identified at the molecular level. Limet et al. [51] found independent evidence for reducing conditions in early liver endosomes sufficient to break disulfide bridges in antibodies, but nothing is known about the molecular mechanism of this reduction. A transmembrane enzymatic activity with low acceptor specificity would be a good candidate and would also be expected to show reduced activity at low temperature.

Endosomal fractions displayed NADH–ferricyanide reductase activity which was stimulated by latency-breaking concentrations of detergent. Activity of NADH–ferricyanide reductase in endosomal fractions has been considered a measure for contamination with other intracellular membranes. Apart from the fact that this enzyme activity can be found in all endomembranes of liver [52–55], assuming only contamination as source of enzyme activity seems to be incorrect for the following reasons: No activation by detergent was seen in a sucrose gradient fraction mainly containing endoplasmatic reticulum. NADH–cytochrome-c reductase activity, another marker for endoplasmic reticulum and outer mitochondrial membranes, has a unilateral orientation in the membrane and showed no detergent stimulation either. We cannot conclude with certainty whether the observed latency applies to acceptor, to donor or to both, since NADH as well as ferricyanide do not cross membranes. Inside-out vesicles from endoplasmic reticulum or Golgi apparatus, expected to show latency for donor and acceptor, are not usually observed [54], and in this case one would expect latency for cytochrome-c reductase, too. If what is observed were acceptor latency, the orientation of the enzyme would correspond to the transplasma-membrane reductase [56] and the dehydroascorbate-stimulated reductase of clathrin-coated vesicles [57]. The former has been shown to react with transferrin-bound iron under appropriate conditions [58], a finding which is, however, not generally agreed upon [59]. As judged from its localization in clathrin-coated vesicles, this enzyme may actually be recycling together with the receptor or may be endogenous to all endocytic membranes providing the necessary reducing equivalents for iron reduction by a transmembrane mechanism.

Iron reduction in the endocytic compartment may be a physiological function of transmembrane pyridinenucleotide oxidoreductase.

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