Subfractionation of hepatic endosomes in Nycodenz gradients and by free-flow electrophoresis

Separation of ligand-transporting and receptor-enriched membranes

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1. The complexity of rat liver endosome fractions containing internalized radioiodinated asialotransferrin, asialo-(alkaline phosphatase), insulin and prolactin was investigated by using free-flow electrophoresis and isopycnic centrifugation in Nycodenz gradients. 2. Two subfractions were separated by free-flow electrophoresis. Both subfractions contained receptors for asialoglycoprotein and insulin. Glycosyltransferase activities were associated with the more electronegative vesicles, whereas 5'-nucleotidase and alkaline phosphodiesterase activities were associated with the less electronegative vesicles. 3. Three subfractions were separated on Nycodenz gradients. Two subfractions, previously shown to become acidified in vitro, contained the ligands. At short intervals after uptake (1–2 min), ligands were mainly in subfraction DN-2 (density 1.115 g/cm^3), but movement into subfraction DN-1 (density 1.090 g/cm^3) had occurred 10–15 min after internalization. Low amounts of glycosyltransferase activities were associated with subfraction DN-2, and 5'-nucleotidase and alkaline phosphodiesterase activities were mainly located in subfraction DN-1. 4. The binding sites for asialoglycoproteins and insulin were distributed towards the higher density range in the Nycodenz gradients, thus indicating a segregation of receptor-enriched vesicles and those vesicles containing the various ligands 10–15 min after internalization. 5. Electron microscopy of the subfractions separated on Nycodenz gradients indicated that whereas the ligand-transporting fractions consisted mainly of empty vesicles (average diameter 100–150 nm), the receptor-enriched component was more granular and smaller (average diameter 70–95 nm). 6. The properties of the endosome subfraction are used to assign their origin to the regions of the endocytic compartment where ligand–receptor dissociation and separation occur.

INTRODUCTION

Mammalian cells have evolved mechanisms for ensuring the rapid internalization and dissociation of receptor–ligand complexes formed initially at the cell surface. The dissociation and sorting of ligands and receptors occurs in acidic endocytic compartments distinguishable from lysosomes (Yamashiro & Maxfield, 1984). Numerous subcellular-fractionation studies of cells and tissues that have internalized radioiodinated ligands have been carried out to identify membrane components comprising the endocytic compartment. Progress has been made towards isolating the components of the hepatic endocytic compartment (endosomes) and towards cataloguing their biochemical properties, especially with respect to the other subcellular organelles historically implicated in receptor-mediated endocytosis, i.e. the lysosomes and the Golgi apparatus (Debanne et al., 1982; Luzio & Stanley, 1983; Quintart et al., 1984; Saermark et al., 1985).

In the present work we have used two radioiodinated asialoglycoproteins and two polypeptide hormones to monitor the subfractionation of endosomes at two early stages after internalization, by using the techniques of free-flow electrophoresis and isopycnic centrifugation in Nycodenz density gradients. Three subfractions were identified with properties compatible with involvement in the endocytosis of ligands and receptor recycling in hepatocytes as described morphologically (Wall et al., 1980; Geuze et al., 1983a, b; Hopkins, 1983).

EXPERIMENTAL

Materials

Asialotransferrin (Type 3; prepared as described by Regoeczi et al., 1979), insulin (pig; Novo Industries, Copenhagen, Denmark) and dog asialo-(alkaline phosphatase) prepared as described by Scholtens et al. (1982) were iodinated by using the iodogen method (Fraker & Speck, 1978). Human ^{125}I-prolactin, of radioimmunoassay quality, was obtained from the Pituitary Hormone Laboratory, St. Bartholomew's Hospital, London. All radiochemicals were obtained from Amersham International, Amersham, Bucks., U.K. Nycodenz was purchased from Nyegaard U.K. Ltd., Birmingham, U.K. Other reagents were purchased from Sigma and British Drug Houses.

Administration of ligands

Livers were removed from two Sprague–Dawley female rats (150–200 g) 1–2 min or 10–15 min after intraportal injection of 0.5 ml of one of the following ligands: ^{125}I-asialotransferrin Type 3 (5 μg), ^{125}I-asialo-(alkaline phosphatase) (25 μg), ^{125}I-insulin (10 μg), ^{125}I-prolactin (25 ng) dissolved in phosphate-buffered saline (Evans & Bruning, 1970), pH 7.4, containing 0.1% bovine serum albumin (Cohn fraction IV).

Preparation of endosome fractions

Liver homogenates were fractionated as described previously (Debanne et al., 1982; Saermark et al., 1985).

After removal of a nuclear pellet (1000 *g* for 10 min) and a mitochondrial–lysosome pellet containing a loose overlay of Golgi-apparatus components (33000 *g* for 8 min), the supernatants were centrifuged for 3½ h at 108000 *g* into 15–40% (w/v) linear sucrose gradients, and components within the density ranges 1.120–1.140 g/cm³ and 1.070–1.120 g/cm³ collected; these density ranges corresponded to the general position of the ligands used at 1–2 min and 10–15 min intervals, respectively, after internalization (Evans *et al.*, 1983; Saermark *et al.*, 1985). The density range where the various ligands were recovered 10–15 min after uptake was analysed in greater detail, as noted in the Figures; however, the sucrose density range where the ligands were located 1–2 min after uptake was also examined. In some instances, the pooled fractions were concentrated to 4–5 ml by vacuum dialysis.

Subfractionation of endosomes

(*a*) **Column chromatography.** Fractions used for free-flow electrophoresis analysis were applied to a Sepharose 2B column (41 cm × 1 cm), equilibrated in 10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA/0.15 M-NaCl, or to a controlled-pore glass column (58 cm × 1 cm; Electro-Nucleonics Inc., Fairfield, NJ, U.S.A.). In some instances, 0.25 M-sucrose was included in the equilibration buffer. Approx. 80–85% of the radioactivity and 20–30% of the protein applied to the columns were eluted as a single peak in the void volume.

(*b*) **Free-flow electrophoresis.** The endosome fractions recovered in the two sucrose-density ranges (see above) were analysed in an Elphor VaP5 (Bender and Hobein, Munich, Germany) free-flow-electrophoresis apparatus. An electrode buffer consisting of 100 mM-acetic acid adjusted to pH 7.2 was used, and the buffer in the separating chamber was 10 mM-triethanolamine/10 mM-acetic acid, pH 7.2, made iso-osmolar with 0.27 M-sorbitol. Endosome fractions were suspended in and dialysed against the separating-chamber buffer at 2–3 mg of membrane protein/ml and injected into the chamber at a rate of 1–2 ml/h. The electrophoretic conditions used were: 1200 V, 130 mA and 6 °C. The chamber-buffer flow rate was 2 ml/h per fraction; 60 fractions were collected and analysed.

(*c*) **Density-gradient separations.** Endosome fractions recovered in the two sucrose-density ranges (see above) were centrifuged for 16 h in continuous gradients constructed of 15–60% (w/v) sucrose solutions or in continuous Nycodenz gradients constructed by mixing 6 ml of 27.6% (w/v) Nycodenz dissolved in water and 6 ml of a 13.8% (w/v) Nycodenz solution dissolved in 8% sucrose and resting on a 1 ml cushion of 70% (w/v) sucrose. Endosome fractions collected from the preparative sucrose gradient were diluted with an equal volume of water before application to the Nycodenz gradients in 14 ml cellulose nitrate tubes and centrifugation at 108000 *g* for 16 h in a Beckman SW.28 rotor. Gradients were unloaded, generally, to yield 20 0.7 ml fractions, and the distributions of radioiodinated ligands, enzymes, ligand-binding and protein content were determined.

Table 1. **Recovery and relative specific activities of radioiodinated ligands and various marker enzymes in the low-density endosome fraction**

Results were obtained by using fractions pooled within the sucrose density range 1.070–1.120 g/cm³ and prepared 10–15 min after injection of radioiodinated ligands into the hepatic vein. Approx. 1 mg of protein/g wet wt. of liver was recovered in this density range; a similar recovery of protein was obtained from the 1.120–1.140 g/cm³ sucrose density range. Results are means (±S.D., where indicated) for the number of determinations indicated in parentheses.

Component in fraction		Recovery from homogenate (%)	Specific activity relative to homogenate	Source
Radioiodinated ligands				
Asialotransferrin type 3		19.4±0.9 (4)	62.0	Debanne *et al.* (1982)
Asialo-(alkaline phosphatase)		9.2 (2)	32.0	The present work
Insulin		7.9±1.1 (4)	25.0	Debanne *et al.* (1982)
Prolactin		16.2 (2)	39.0	The present work
Cyanopindolol (β-adrenergic ligand)		16.0 (2)	29.3	Hadjiivanova *et al.* (1984)
Marker enzymes				
Monensin-activated Mg²⁺-ATPase		—	60.0 ⎫	Saermark *et al.* (1985)
Galactosyltransferase ⎱	Golgi	⎰ 21.0 (2)	34.5 ⎭	
Sialyltransferase ⎰	apparatus	⎱ 9.5 (2)	31.0	The present work
5′-Nucleotidase ⎱		⎰ 6.7±2.4 (3)	16.5 ⎫	Saermark *et al.* (1985)
Alkaline phosphodiesterase		2.5±0.4 (3)	6.5 ⎭	
Na⁺/K⁺-activated Mg²⁺-ATPase	Plasma	—	5.2	The present work
Adenylate cyclase (basal)	membranes	1.4 (2)	0.1	Hadjiivanova *et al.* (1984)
Leucine aminopeptidase		0.3 (2)	0.1 ⎫	The present work
γ-Glutamyl transpeptidase ⎭		⎱ 0.4 (2)	0.3 ⎭	
Acid phosphatase ⎱		⎰ 1.0 (2)	2.2 ⎫	Debanne *et al.* (1982)
β-Galactosidase ⎭	Lysosomes	⎱ 0.09 (2)	2.1 ⎭	
NADPH–cytochrome *c* reductase	Endoplasmic reticulum	1.8 (2)	1.74	The present work

Enzyme and ligand-binding assays

Enzyme activities were measured as described previously (Debanne *et al.*, 1982; Saermark *et al.*, 1985). Asialoglycoprotein binding was measured as described by Pricer & Ashwell (1976), with asialo-orosomucoid. Insulin binding was determined as described by Evans *et al.* (1973). Protein was measured with the Folin–Ciocalteu's reagent, except in samples collected from Nycodenz gradients, when the Coomassie Blue dye-binding procedure (Bio-Rad Laboratories kit) was used, and in the free-flow-electrophoresis samples, when a micro-tannin turbidometric method (Mejbaum-Katzenellenbogen & Dobryszycha, 1959) was used.

RESULTS

Table 1 summarizes the properties of the endosome fraction used in the subfractionation work reported. The presence at short intervals after internalization of radioiodinated ligands at concentrations manyfold higher than in the liver homogenate and the identification of a monensin-activated Mg^{2+}-ATPase that may account for the ATP-dependent acidification emerge as linked markers for the fraction. Markers for other subcellular components were either present at low amounts or at concentrations that rendered it difficult to assess whether they were contaminants or a reflection of the movement of membrane components from the plasma membrane during receptor-mediated uptake of ligands. To resolve this question, the endosome fractions were examined by using two techniques that resolve membranes according to surface charge or density.

Free-flow-electrophoretic separations

Analysis of the endosome fraction indicated that the membranes emerged as a single asymmetrical peak (Fig. 1*a*). Analysis of the distribution of four radioiodinated ligands in endosome fractions isolated 10–15 min after ligand administration showed two major overlapping peaks (Figs. 1*b*–1*e*, fractions 22–34). Further peaks of ligands (asialotransferrin, prolactin) located in regions relatively protein-free at the electronegative side of the separating chamber were presumed to contain free undegraded ligand lost from the vesicles before and during the free-flow electrophoresis; the non-protein-associated ligand peaks varied from experiment to experiment. Analysis of the distribution of the galactosyl- and sialyl-transferase activities retained in the fraction injected into the apparatus (Figs. 1*f*, 1*g*) showed that they were coincident with the ligand-transporting components on the electropositive side of the separating chamber, whereas the peaks of 5′-nucleotide and alkaline phosphodiesterase activities were coincident with the other components migrating with the major protein components. Sialic acid present in the endosome membranes may be a major contributor to this electronegativity (Evans & Hardison, 1985).

Fig. 2 shows, in a different experiment, the distribution of binding sites for asialoglycoproteins (asialo-orosomucoid) or insulin. Again it was apparent that the distribution of the binding sites of two ligands identified two major endosomal subfractions. In separate experiments, these two peaks of receptors were shown to be coincident, relative to the protein peak, with the two peaks of iodinated ligands and enzymes identified in Fig.
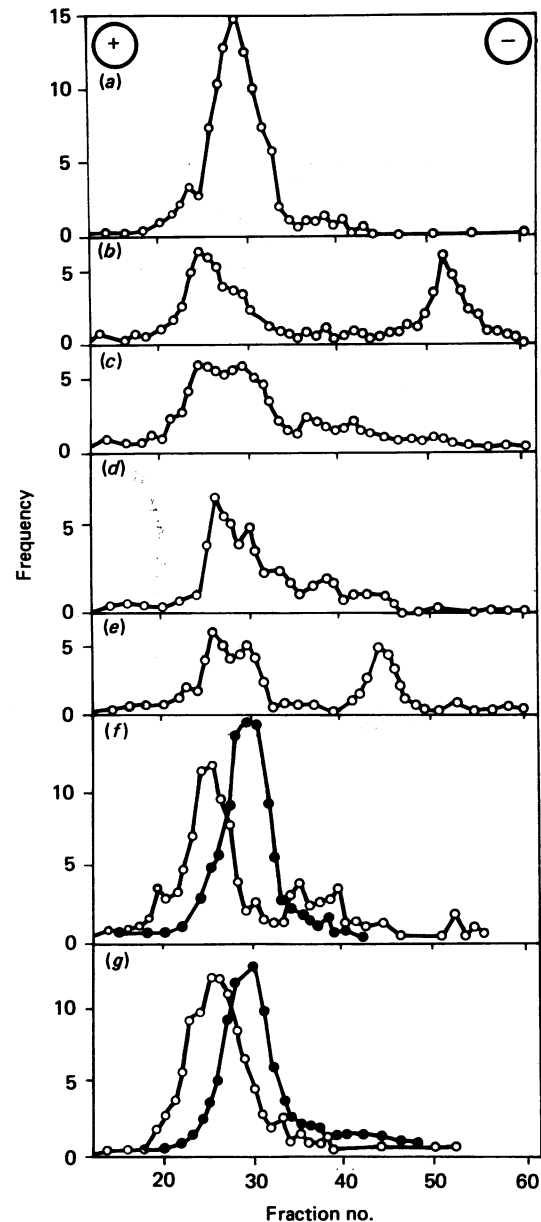

Fig. 1. Distribution of various ^{125}I-labelled ligands and enzymes in the endosome fraction separated by free flow electrophoresis

See the Experimental section for details. The results are expressed as the frequency of distribution of the components recovered from the apparatus. Livers were homogenized 15 min after injection of ^{125}I-labelled ligands into the hepatic portal vein, and components in the sucrose density range 1.070–1.117 g/cm^3 after exclusion chromatography were injected (3–5 ml) into the apparatus. (*a*) Protein; (*b*) ^{125}I-asialotransferrin; (*c*) ^{125}I-insulin; (*d*) ^{125}I-asialo-(alkaline phosphatase); (*e*) ^{125}I-prolactin; (*f*) ○, galactosyltransferase; ●, alkaline phosphodiesterase; (*g*) ○, sialyltransferase; ●, 5′-nucleotidase.

1. Membranes collected from the sucrose gradient where the internalized ligands were located 1–2 min after injection into the portal vein (density range in sucrose 1.12–1.14 g/cm^3) were also analysed in the free-flow-electrophoresis apparatus, and the results (not shown)
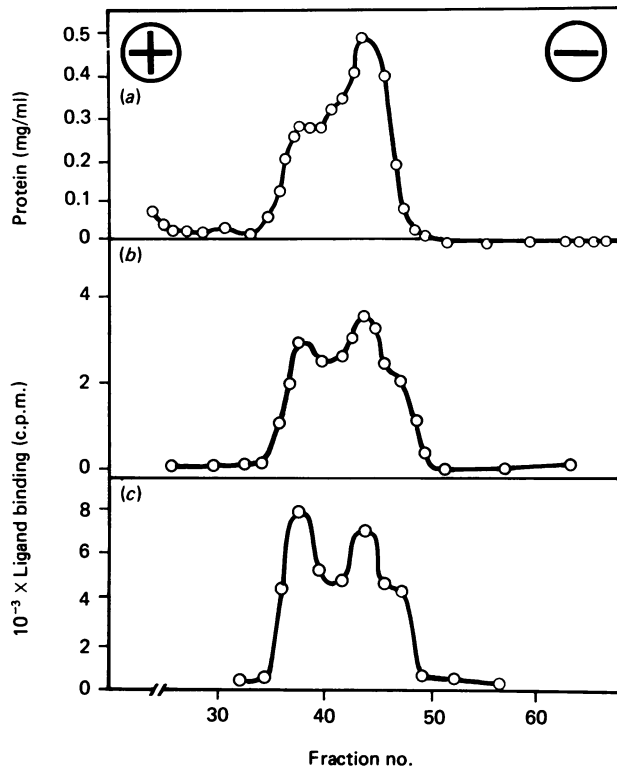
Fig. 2. Distribution of (a) protein, (b) asialoglycoprotein-binding sites and (c) insulin-binding sites in an endosome fraction separated by free-flow electrophoresis

See the Experimental section for further details.

also indicated a similar separation into two peaks of radioiodinated ligands and enzyme activities.

Density-gradient fractionation

The ligand-transporting vesicles, collected from the sucrose gradient, were subjected to overnight centrifugation in sucrose and Nycodenz gradients. Preliminary experiments involving centrifugation for 18 h at 98 500 g in 15–50% (w/v) sucrose gradients indicated that only a partial separation of the ligand-transporting and galactosyltransferase-containing components was achieved (results not shown). A more clear-cut separation of internalized radio-ligands and galactosyltransferase was obtained by overnight centrifugation in iso-osmotic Nycodenz gradients, prompting a full examination of the distribution in the gradients of various radio-ligands administered 1–2 min and 10–15 min after injection into the portal vein, as well as marker enzymes and receptors for asialoglycoproteins and insulin.

Fig. 3 shows the distribution in the Nycodenz gradients of two radioiodinated asialoglycoproteins and two polypeptide hormones internalized by liver, 10–15 min after injection into the portal vein. The ligands were located in two regions at the light end of the Nycodenz gradient; these components corresponded to densities of 1.090 (DN-1) and 1.115 g/cm³ (DN-2). At 1–2 min after uptake, when components in the sucrose density range 1.12–1.14 g/cm³ were examined, asialotransferrin and insulin were located mainly in the middle of the gradient in fraction DN-2, indicating that during endocytosis the ligands moved between membranes of the endocytic

networks recovered in fraction DN-2 (early endosomes) to components of lower density recovered in fraction DN-1 ('late' endosomes) (Figs. 3a and 3c).

Examination of the distribution of enzymes (Fig. 3, panels f–j) showed that 5'-nucleotidase and alkaline phosphodiesterase activities associated with the endosome fraction (Table 1) were recovered in fractions DN-1 and DN-2, a distribution similar to that reported for a monensin-activated Mg^{2+}-ATPase (Saermark et al., 1985). The galactosyl- and sialyl-transferase activities were confined mainly to fraction DN-2. Finally, NADP–cytochrome c reductase activity, an endoplasmic-reticulum marker, was more generally distributed across the gradients.

Analysis of the distribution of asialoglycoprotein- and insulin-binding sites (Fig. 4) showed that these were located mainly in components in fractions D-R and DN-2 on the Nycodenz gradients. The distribution of binding sites thus contrasted with that of ligands, for these were located mainly in fraction DN-2 1–2 min after uptake (Fig. 3) and in fractions DN-1 and DN-2 10–15 min after uptake.

Morphological features of the fractions

The endosome fractions, when thin sections were examined by electron microscopy, contained an assortment of vesicular membrane profiles. In the separations obtained by free-flow electrophoresis, it was noted that multivesicular bodies were located mainly in the major electropositive peak, but that small granular vesicular components (average diameter 70–95 nm) were the major components in both the peaks separated. Morphological distinctions between the three major fractions separated in Nycodenz gradients were more clear-cut. Thus, as shown in Fig. 5, fraction DN-1 contained large numbers of vesicles (average diameter 100–150 nm) with little visible internal content, and fraction DN-2 contained, in addition, a greater proportion of multivesicular structures. It was also evident that the smaller granular vesicular components were present in fraction DN-2, but were more concentrated towards the bottom of the Nycodenz gradient in fraction D-R. No other morphologically identifiable components were seen, with the exception of occasional free ribosomes and ferritin-like particles that were located at the bottom of the gradients, imparting a reddish-brown appearance.

DISCUSSION

Resolution and analysis of ligand-containing low-density components

The present work, taken together with the analysis of lipids and proteins reported (Evans & Hardison, 1985), allows the origin of the membrane components now resolved to be discussed with reference to the morphological features of the endocytic component. The endocytic networks are usually described as a series of tubular-vesicular networks containing detaching vesicles and multivesicular bodies and retreating into the cytoplasm from coated-pit regions of the plasma membrane (Wall et al., 1980; Geuze et al., 1983a, b; Hopkins, 1983). Little is known about the outward leg along which many receptors and those ligands that undergo diacytosis (e.g. asialotransferrin, transferrin) are returned to the plasma membrane.
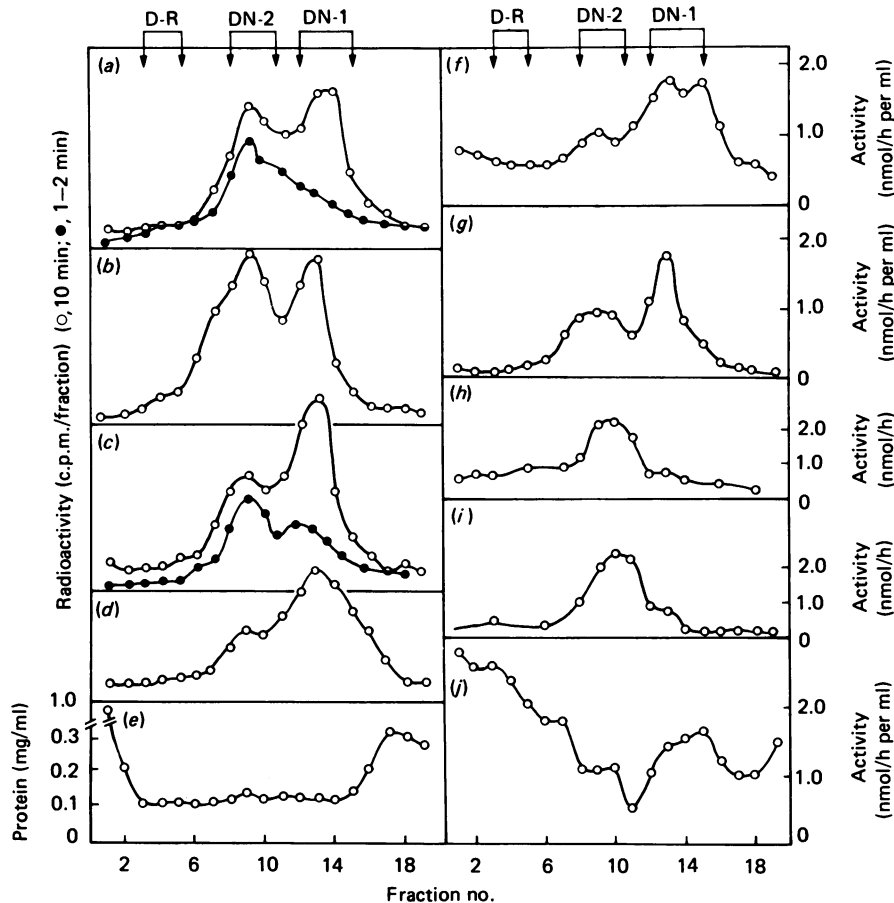
Fig. 3. Distribution of various ^{125}I-labelled ligands and marker enzymes in the endosome fractions separated in Nycodenz gradients as described in the Experimental section

(a) ^{125}I-asialotransferrin in endosome fractions prepared 1–2 min (●; density range 1.120–1.140 g/cm³ in sucrose gradients) or 10 min (○; density range 1.070–1.120 g/cm³) after injection into the hepatic portal vein; (b) ^{125}I-asialo-(alkaline phosphatase); (c) ^{125}I-prolactin in endosome fractions prepared 1–2 min (●) or 10 min (○) after injection into the portal vein; (d) ^{125}I-insulin; (e) protein; (f) 5′-nucleotidase; (g) alkaline phosphodiesterase; (h) galactosyltransferase; (i) sialyltransferase; (j) NADP–cytochrome c reductase. Enzyme determinations were carried out on the endosome fraction recovered in sucrose-density range 1.070–1.120 g/cm³ applied to the Nycodenz gradient. For further details see the Experimental section. The pooled fractions corresponded to the following density positions: D-R, interface between the Nycodenz gradient and the sucrose cushion; DN-1 and DN-2, refractive-index ranges 1.355–1.364 and 1.365–1.375 respectively, and peak densities 1.090 and 1.115 g/cm³ respectively.

The enzymic properties of the subfractions, especially DN-1 and DN-2, now separated in Nycodenz gradients, further reinforce the conclusion that the components now isolated from the endocytic network have a biochemical identity which is distinct from lysosomes, plasma membranes and the Golgi apparatus. The low activities of leucine aminopeptidase, a bile-canalicular plasma-membrane marker enzyme (Roman & Hubbard, 1984), of Na^+/K^+-activated Mg^{2+}-ATPase, a sinusoidal–basolateral plasma-membrane marker enzyme (Poupon & Evans, 1979), and of adenylate cyclase, especially the ligand-induced activation of this enzyme (Hadjiivanova et al., 1984), are further evidence of the low recovery of membrane fragments originating from the various domains of the plasma membrane. The presence of 5′-nucleotidase and alkaline phosphodiesterase activities, especially in subfraction DN-1 and in the components of the protein peak nearest the electronegative electrode in the free-flow-electrophoresis separation, probably reflect

the internalization and recycling of these glycoprotein enzymes. This possibility is supported by observations of intracellular pools of 5′-nucleotidase in hepatocytes (Luzio & Stanley, 1983) and fibroblasts (Widnell et al., 1982).

The persistence of glycosyltransferase activities associated mainly with subfraction DN-2 and the subfraction separated by free-flow separation is more difficult to account for. Although these enzymes are recognized as specific, although not necessarily exclusive, biochemical markers for the Golgi apparatus, no characteristic Golgi cisternal profiles could be identified in the fractions isolated. Few lipoprotein-filled vacuoles were seen in these fractions. Analysis of the phospholipid and cholesterol composition of the subfractions DN-1 and DN-2 showed that both fractions resembled plasma membranes more closely than Golgi fractions (Evans & Hardison, 1985), and a comparison of the lipid fluidity of the parent endosome fraction also showed a similarity to plasma
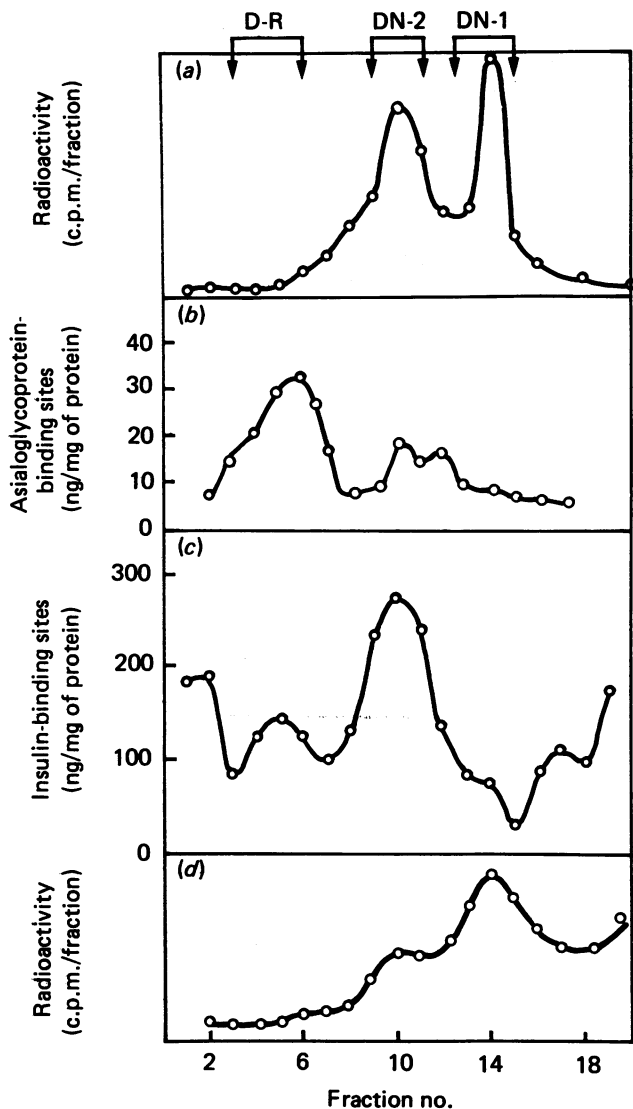
Fig. 4. Distribution of ^{125}I-labelled ligands and their respective binding sites in Nycodenz gradients

(a) ^{125}I-asialotransferrin; (b) asialoglycoprotein-binding sites; (c) insulin-binding sites; (d) ^{125}I-insulin. For further details see the Experimental section and legend to Fig. 3.
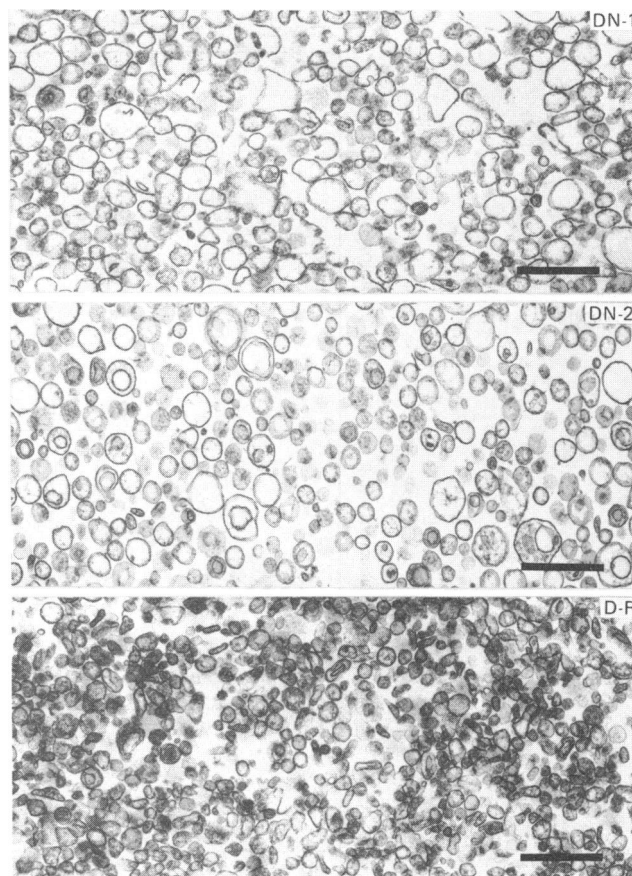

Fig. 5. Electron micrographs of the three endosomal subfractions DN-1, DN-2 and D-R (Fig. 3) recovered from the Nycodenz density gradients

Bars = 0.5 nm.

membranes rather than to Golgi fractions (Whetton et al., 1983). Using an endosome fraction isolated from rat liver by an equilibrium-density-shift method, Quintart et al. (1984) also showed that endosome vesicles, shown morphologically to enclose ligands, contained low activities and recoveries of galactosyltransferase and 5'-nucleotidase. Furthermore, an hepatic endosome fraction prepared by the present procedure was shown, by immunoadsorption to beads coated with antibodies to the hepatic lectin, to separate into ligand-transporting components bound to the beads, with sialyltransferase activity remaining in unbound components (Debanne et al., 1984). Endosomes prepared from human KB cells were shown to be depleted in marker enzymes of plasma membranes, lysosomes and the Golgi apparatus (Dickson et al., 1983). Thus endosome membranes originate from a biochemically distinctive subcellular compartment.

Separation of ligand- and receptor-containing components

Although the free-flow-electrophoresis technique indicated the presence of at least two populations of ligand-containing components, it did not separate the receptor-bearing components from ligand-transporting components. However, the Nycodenz gradients, as well as separating two ligand-containing fractions, also showed that components with binding sites for asialoglycoproteins and insulin were located in denser vesicles recovered in subfractions DN-R and DN-2. The topography of the binding sites on the components located at higher density was not investigated in the present study. However, there is evidence that membranes containing exposed asialoglycoprotein receptors are recovered in hepatic endosome fractions, as shown by the use of proteolytic enzymes and detergents (Debanne et al., 1982) and immunoabsorption (Debanne et al., 1984). Morphological analyses of the topographical distribution of ferritin and gold-labelled epidermal growth factor (McKanna et al., 1979) and transferrin (Hopkins & Trowbridge, 1983; Harding et al., 1983), respectively, have identified receptor-enriched membranes in trabecular and multivesicular bodies, and these, after disruption and release during subcellular fractionation, may correspond to the components separated in the Nycodenz gradients after isopycnic centrifugation. The smaller size of the
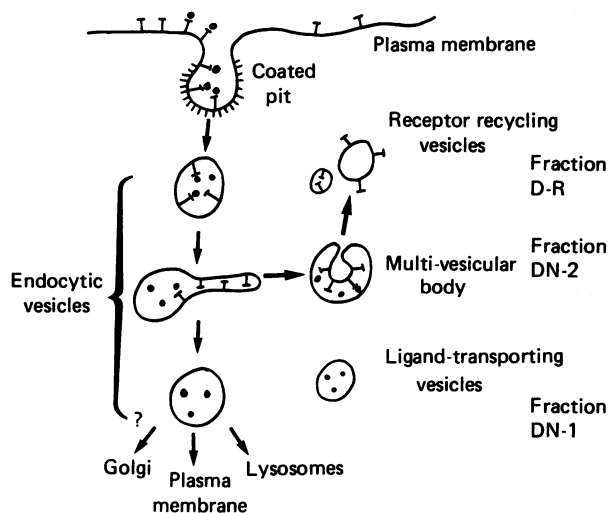
Fig. 6. Diagrammatic representation of the hepatic endocytic compartment in relation to the origin of the three endosome subfractions separated on the Nycodenz gradients (Fig. 3)

Ligand (●)-receptor (T) complexes, concentrated in coated pits, are transferred into the endocytic compartment that is fragmented during homogenization of the liver to yield (1–2 min after uptake) vesicles containing receptors and ligands, recovered in subfraction DN-2, and (10–15 min after uptake) vesicles containing ligands originating from regions where uncoupling and separation of ligand–receptor complexes has occurred; these were recovered mainly from subfraction DN-1. Fraction D-R, containing asialoglycoprotein- and insulin-binding sites, but little bound ligand, probably comprises components of the outward leg of the endocytosis pathway and/or from the membrane where the pools of intracellular receptors are located. The topography of the receptors in the recovered subcellular fraction is discussed in the text.

components (diameter 70–95 nm) compared with the larger vesicles (diameter 90–150 nm) may explain their lower sedimentation rate, and thus coincidence with ligand-transporting components in the initial sucrose-density-gradient separation step (Saermark et al., 1985). However, the longer centrifugation times used to fractionate the endosomes in Nycodenz gradients resulted in the smaller vesicles, that were in a majority in the parent fraction, equilibrating at the higher density, presumably reflecting their higher protein/lipid weight ratios. These separations contrast with those reported by Kindberg et al. (1984), who showed, using Nycodenz gradients, that asialoglycoproteins internalized by liver were associated initially with small vesicles sedimenting at low speed, and later with fast-sedimenting larger vesicles. Vesicles with similar properties to those in fraction D-R may also account for the asialoglycoprotein receptors present in liver lysosome-enriched fractions (Tanabe et al., 1979).

Delineating the endocytic pathway

The time-dependence of the distribution on density gradients of the internalized ligands, combined with the biochemical and morphological features of the isolated fractions, permit the likely origin of components from the endocytic networks of components in the three endosome subfractions to be assessed (Fig. 6). Endocytosis may be divided into an inward leg, traversed by ligand–receptor complexes, and an outward leg traversed, in the main, by the receptor being returned to the plasma membrane. A receptor in the outward leg may not necessarily be one newly dissociated from a ligand, but can arise from pools of internal receptors. The vesicles recovered in fraction DN-2 in Nycodenz gradients ('early' vesicles) may be derived from a region of the endocytic networks where a pH-induced separation and segregation of the ligand–receptor complexes occurs. Fraction DN-1, which also contains a proton pump (Saermark et al., 1985), contains vesicles arising from a region of the endocytic networks where the ligand is present, but has separated from receptor-containing membrane fragments that may arise from the receptor-enriched rims of the endocytic compartments (Geuze et al., 1983a); these regions may vesiculate to yield small, denser, components now recovered in fraction D-R. Since asialotransferrin was used at concentrations in which it is returned from the endosome compartment to the cell surface (Regoeczi et al., 1982), it might be expected that the ligand would also be associated with components in this fraction. However, this apparent anomaly can be explained by invoking the presence in this fraction of the components bearing uninvolved pools of receptors associated with or contiguous to the compartment where ligand endocytosis was active, thereby diluting out the radio-ligand concentrations. It has been calculated that 90% of the hepatocyte's receptors are present in intracellular components (Ashwell & Harford, 1982; Schwartz, 1984), thereby ensuring a large excess of intracellular ligand-free receptors.

In conclusion, the present work has identified three biochemically distinctive component parts of the hepatic endocytic network, two of which contain ligand and a further high-density receptor-enriched fraction. The results are consistent with the events occurring in the endocytic networks, namely dissociation of ligand–receptor complexes, the independent sorting of ligands and receptors, and the presence of intracellular pools of receptors that are possibly involved in receptor recycling and the regulation of the number of receptors at the cell surface.

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