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Rapid subcellular fractionation of the rat liver endocytic compartments involved in transcytosis of polymeric immunoglobulin A and endocytosis of asialofetuin

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The distributions of two endocytosed radiolabelled ligands (polymeric immunoglobulin A and asialofetuin) in rat liver endocytic compartments were investigated by using rapid subcellular fractionation of post-mitochondrial supernatants on vertical density gradients of Ficoll or Nycodenz. Two endocytic compartments were identified, both ligands being initially associated with a light endocytic-vesicle fraction on Ficoll gradients, asialofetuin then accumulating in denser endosomes before transfer to lysosomes for degradation.

INTRODUCTION

Understanding the biochemical events involved in sorting and movement on endocytic routes would be greatly facilitated by the rapid analytical separation of endocytic vesicles from such pathways. Asialofetuin (ASF) and polymeric immunoglobulin A (pIgA) are both endocytosed by rat hepatocytes, and may be iodinated without affecting their uptake and intracellular fate (Schiff *et al.*, 1984; Van Berkel *et al.*, 1986). ASF is endocytosed after binding to a galactose-specific receptor (Tolleshaug *et al.*, 1977, 1980), passes through an acid endosome compartment known as CURL (the compartment of uncoupling receptor and ligand; Geuze *et al.*, 1984) and then, like other asialoglycoproteins, is degraded in lysosomes (Limet *et al.*, 1985). In contrast, pIgA binds and becomes disulphide-bonded to a blood-sinusoidal surface receptor and is then endocytosed and passes through CURL before being released from the bile-canalicular surface of the hepatocytes as a result of proteolytic cleavage of the receptor (Geuze *et al.*, 1984; Mostov & Simister, 1985). In the present experiments, rat livers were homogenized at different times after intravenous injection of radiolabelled ligands. Post-mitochondrial supernatants were fractionated by isopycnic centrifugation on vertical 1–22% (w/v) Ficoll gradients, which allow separation of endosome-derived membranes within 2 h (Branch *et al.*, 1986*a*,*b*), in contrast with the previous requirement for sequential overnight Ficoll and Metrizamide gradient centrifugation steps to isolate pIgA-containing endocytic vesicles (Mullock *et al.*, 1983, 1987). Analysis of endocytosed ligand transfer to lysosomes was carried out on 0.25 M-sucrose–45% (w/v) Nycodenz gradients.

EXPERIMENTAL

Rat pIgA (Orlans *et al.*, 1978) and ASF (Sigma, type I) were radiolabelled as described by Mullock *et al.* (1983).

Radiolabelled ligands were administered separately or together as a single bolus injection (200 μl in PBS) into the femoral vein of anaesthetized (Hypnorm; Janssen Pharmaceuticals, Oxford, U.K.) male Wistar rats (200–250 g).

When appropriate, colchicine (50 mg/ml in PBS; 80 mg/kg body wt.) or leupeptin (10 mg/ml; 20 mg/kg) was injected intravenously 1 h before ligand administration.

At fixed times after ligand administration, the livers were perfused via the inferior vena cava with cold 0.25 M-sucrose/10 mM-Tes, pH 7.4, and 10 g of liver was homogenized in 30 ml of 0.25 M-sucrose/10 mM-Tes/1 mM-$MgCl_2$ (pH 7.4) in a Potter–Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 1100 ***g***, generating a post-mitochondrial supernatant, 5 ml samples of which were subsequently fractionated on density gradients of Ficoll or Nycodenz (Nycomed, Birmingham, U.K.) in a vertical rotor (Beckman, VT_i 50) centrifuged for 1 h at 206000 ***g***. After leupeptin administration, the homogenate was centrifuged for 10 min at 600 ***g*** to generate a post-nuclear supernatant, since leupeptin makes lysosomes denser (Dunn *et al.*, 1979), and many were lost after a 10 min centrifugation at 1100 ***g***. Each Ficoll gradient was prepared as a 30 ml linear gradient of 1–22% (w/v) Ficoll containing 0.25 M-sucrose, 10 mM-Tes and 1 mM-EDTA, pH 7.4, on a 5 ml cushion of 45% (w/v) Nycodenz containing 10 mM-Tes and 1 mM-EDTA, pH 7.4. Each Nycodenz gradient was prepared as a 30 ml linear gradient from 0.25 M-sucrose/10 mM-Tes/1 mM-EDTA, pH 7.4, to 45% Nycodenz containing 10 mM-Tes and 1 mM-EDTA, pH 7.4. After centrifugation the gradients were pumped off and samples collected, starting from the bottom of the gradient.

Radioactive-ligand distribution on gradients was assessed by γ-counting of samples at 70% efficiency when only ^{125}I-labelled ligand was present, and 75% (for ^{125}I), 50% (for ^{131}I) when both ^{125}I- and ^{131}I-labelled ligands were present on the same gradient. All other assays were as previously described (Luzio & Stanley, 1983; Mullock *et al.*, 1983).

Abbreviations used: pIgA, polymeric immunoglobulin A; ASF, asialofetuin; PBS, phosphate-buffered saline (0.145 M-NaCl/7.5 mM-Na_2HPO_4/2.5 mM-NaH_2PO_4), pH 7.4.

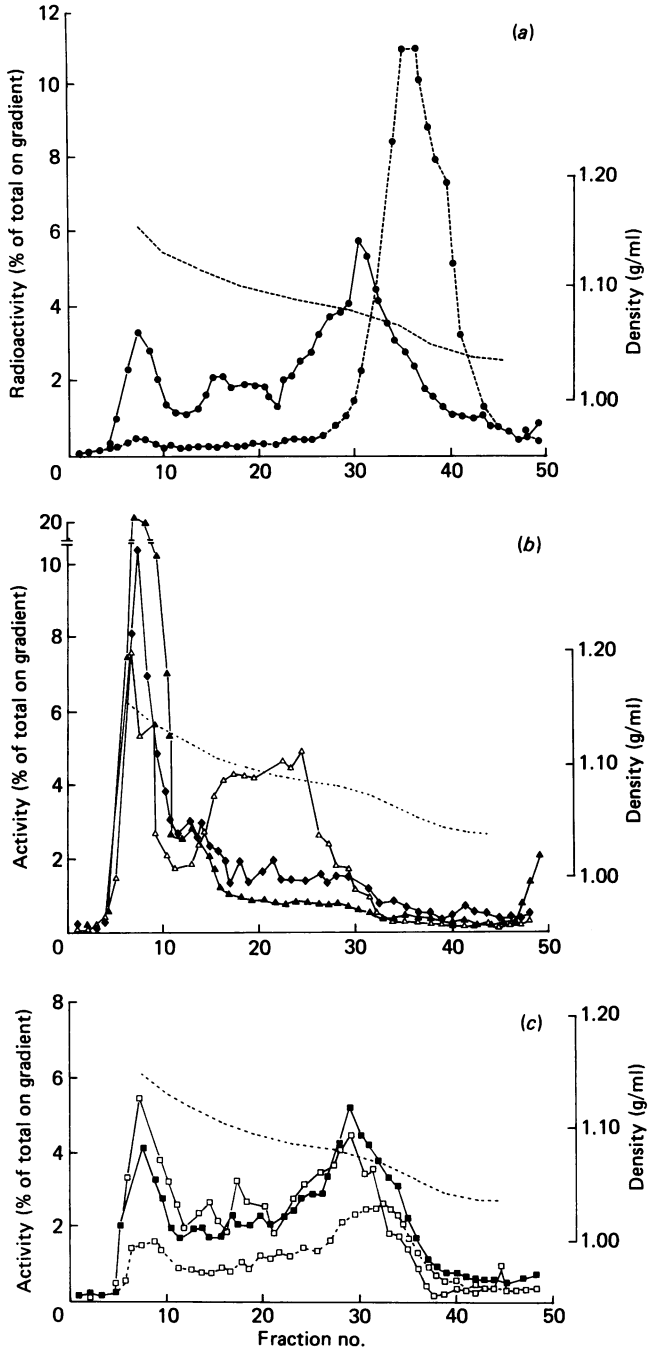

Fig. 1. Distribution of ^{125}I-pIgA and marker enzymes after isopycnic centrifugation on Ficoll gradients in a vertical rotor

Liver post-mitochondrial supernatants were loaded throughout, except for the ^{125}I-pIgA-labelled blood-sinusoidal plasma membrane in (*a*) prepared as described in Mullock *et al.* (1983). All gradients are 1–22% (w/v) Ficoll gradients on a Nycodenz cushion (----, density). Representative profiles are shown in each case. (*a*) Distribution of 30 min-endocytosed ^{125}I-pIgA (●——●) compared with ^{125}I-pIgA-labelled blood-sinusoidal plasma membrane (●----●). Separate gradients were centrifuged, the ^{125}I-distribution profiles being combined for convenience. With endocytosed pIgA, 58% of the homogenate radioactivity was in the post-mitochondrial supernatants, and 72600 c.p.m. was loaded on to the gradient. (*b*) Distribution of succinate dehydrogenase (◆), glucose 6-phosphatase (△) and β-*N*-acetylglucosaminidase (▲). The proportions of homogenate activities in the post-mitochondrial supernatant and the gradient recoveries (in parentheses) for marker enzymes were succinate dehydrogenase 14% (73%), glucose 6-phosphatase 53% (120%), and β-*N*-acetylglucosaminidase 68% (101%). (*c*) Distribution of 5′-nucleotidase. Total activity with 1% Triton X-100 (■——■) and without Triton (□----□) were measured and latent 5′-nucleotidase (□——□) was calculated as the difference between them. The proportions of the homogenate activity in the post-mitochondrial supernatant and the recoveries (in parentheses) were: +Triton 46% (74%), no Triton 44% (66%) and latent 5′-nucleotidase 41% (89%).

RESULTS

Low-density rat liver endocytic vesicles containing ^{125}I-pIgA have previously been identified on Ficoll gradients after overnight isopycnic centrifugation in swing-out rotors (Mullock *et al.*, 1983). In the present experiments it was found that much more rapid centrifugation for 1 h on similar gradients in a vertical rotor provided good separation from blood-sinusoidal plasma membrane (Fig. 1*a*), lysosomal and mitochondrial marker enzymes, and the bulk of glucose 6-phosphatase, an endoplasmic-reticulum marker (Fig. 1*b*). In contrast, the distribution of endocytosed ^{125}I-pIgA on the gradient closely followed the distribution of latent 5′-nucleotidase activity, but was not as broadly distributed as total 5′-nucleotidase activity (Fig. 1*c*).

The availability of vertical-Ficoll-gradient centrifugation to separate endocytic vesicles from rat liver allowed the analysis of the time courses of internalization of pIgA and of ASF (Fig. 2). These ligands were respectively labelled with ^{125}I and ^{131}I and injected together into rats at fixed times before the liver was homogenized. At early time points (1–3 min) after injection, the distribution of ^{125}I-pIgA on the Ficoll gradients was consistent with some being bound to the blood-sinusoidal plasma membrane and some in the light-endocytic-vesicle fraction (Figs. 2*a* and 2*b*). Between 5 and 30 min after injection the ^{125}I-pIgA was associated less with the blood-sinusoidal plasma membrane and increasingly with denser positions on the gradient. Throughout this period the majority of ^{125}I-pIgA was in the light-endocytic-vesicle peak (Figs. 2*c*–2*f*). In contrast with the Ficoll-gradient distribution of ^{125}I-pIgA, ^{131}I-ASF was found in the light-endocytic-vesicle peak only at early times (1–2 min) after injection. By 5 min the majority of ^{131}I-ASF was at a denser position on the gradient where some (< 20%) ^{125}I-pIgA also ran. By 30 min, low-density ^{131}I radioactivity was seen on the gradient, consistent with degradation of ^{131}I-ASF having occurred (Fig. 2*f*).

Separation of endocytosed radioactive ligands by isopycnic centrifugation on Ficoll density gradients showed some radioactivity running at the boundary of the Ficoll density gradient and the Nycodenz cushion (Fig. 2). The coincidence of this radioactivity with β-*N*-acetylglucosaminidase activity (Fig. 1*b*) suggested that it might be in lysosomes. This possibility was further analysed by isopycnic centrifugation of the post-mitochondrial supernatants on 0.25 M-sucrose/45% Nycodenz, which clearly separates the peak of β-*N*-acetylglucosamindase activity from endocytosed ligands (Fig. 3). No distinction of different endocytic

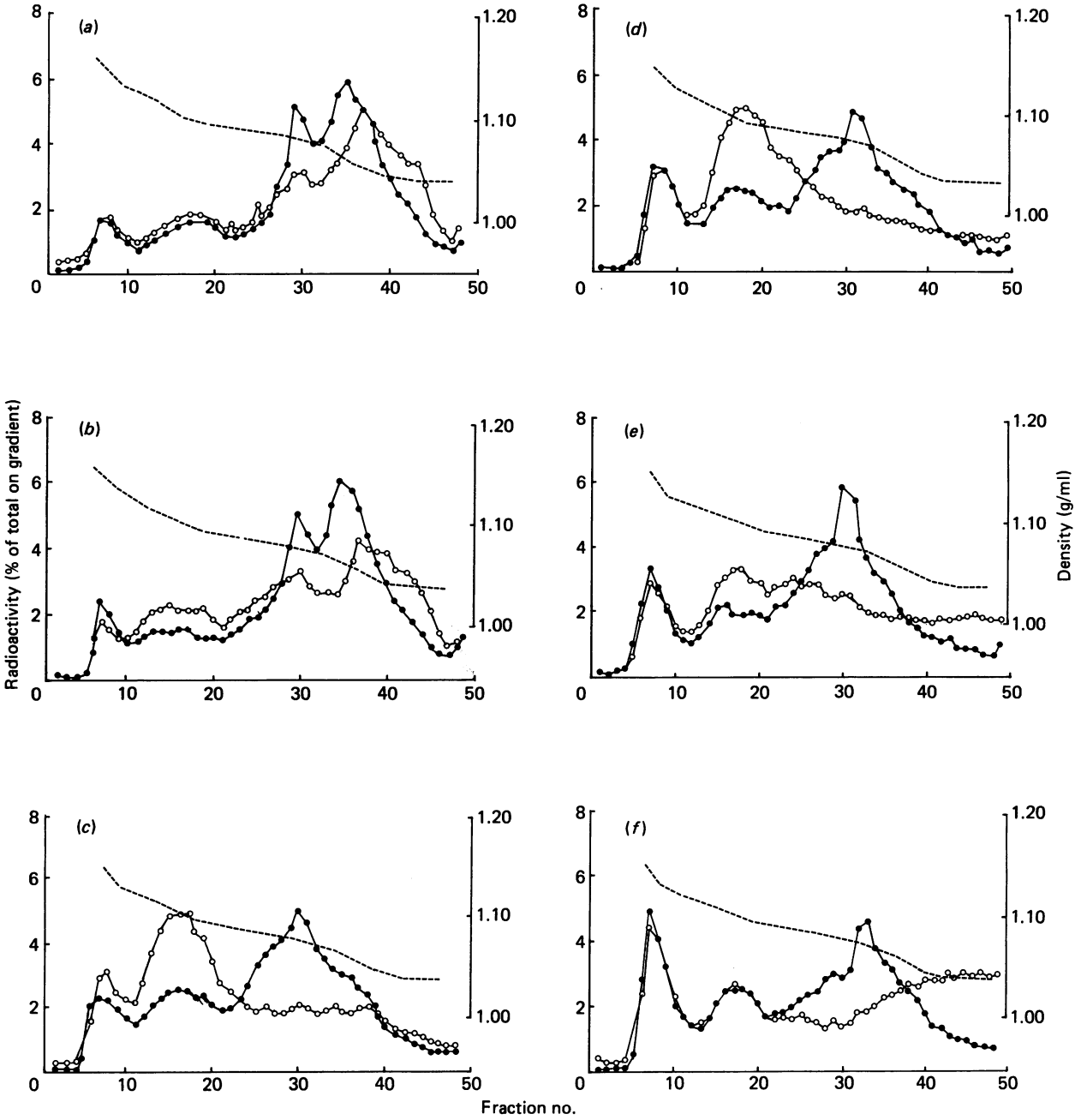

Fig. 2. Distributions of ^{125}I-pIgA and ^{131}I-ASF after isopycnic centrifugation on Ficoll gradients at increasing times after intravenous injection

Livers were taken 1–30 min after ligand injection and post-mitochondrial supernatants were loaded on to Ficoll gradients (----, density). Representative gradients at different times are shown: ●, ^{125}I; ○ ^{131}I. The proportion of homogenate radioactivity in the post-mitochondrial supernatant and the total radioactivity (c.p.m.) loaded on to each gradient in parentheses were as follows: (*a*) 1 min, ^{125}I 50% (54550), ^{131}I 59% (26325); (*b*) 2 min, ^{125}I 53% (100675), ^{131}I 74% (80375), (*c*) 5 min, ^{125}I 52% (126438), ^{131}I 62% (52925); (*d*) 10 min, ^{125}I 58% (78126), ^{131}I 66% (218900); (*e*) 20 min, ^{125}I 58% (72600), ^{131}I 70% (121926); (*f*) 30 min, ^{125}I 61% (71250), ^{131}I 72% (50700).

vesicles was observed on the Nycodenz gradients. By 30 min after internalization of ^{131}I-ASF and ^{125}I-pIgA, approx. 10% of ^{131}I radioactivity and < 10% of ^{125}I radioactivity on the Nycodenz gradient ran as a shoulder in the same region of the gradient as β-*N*-acetylglucosaminidase. In agreement with the data from the Ficoll gradient, low-density ^{131}I radioactivity was observed in the Nycodenz gradient 30 min after ^{131}I-ASF internalization. Clearer evidence that ASF degradation occurred in classical lysosomes after passage through the endocytic compartments was obtained from experiments in which rats were pre-treated with leupeptin, an inhibitor of lysosomal proteinases (Dunn *et al.*, 1979), before ligand injection and liver homogenization. Post-nuclear liver supernatants from leupeptin-treated rats were subjected to isopycnic centrifugation on the Nycodenz gradient 30 min after injection of ^{125}I-pIgA and ^{131}I-ASF. The gradient radioactivity coincident with β-*N*-acetylglucos-

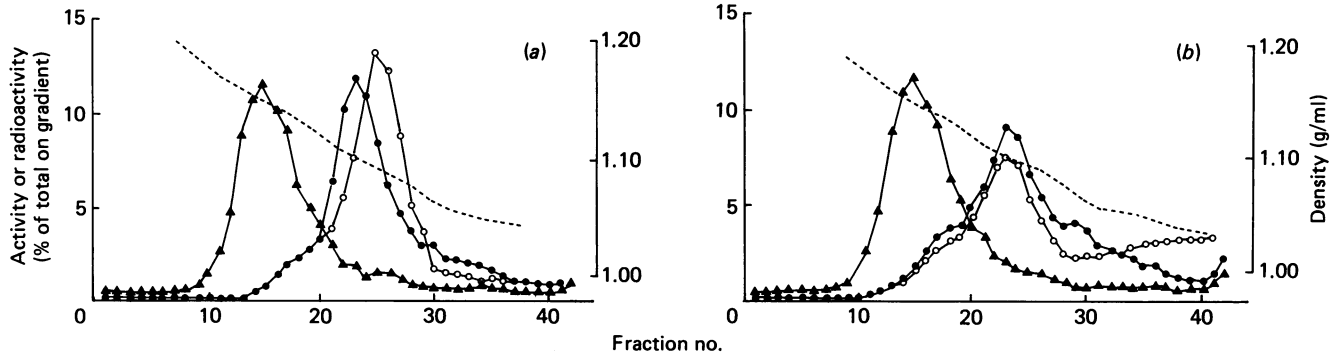

Fig. 3. Distribution of ^{125}I-pIgA and ^{131}I-ASF after isopycnic centrifugation on Nycodenz gradients

Livers were taken 10 min (*a*) and 30 min (*b*) after intravenous ligand injection, and post-mitochondrial supernatants were loaded on to Nycodenz gradients. Representative gradients are shown: ●, ^{125}I; ○, ^{131}I; ▲, *β*-*N*-acetylglucosaminidase. The proportions of homogenate radioactivity in the post-mitochondrial supernatant and the total radioactivity (c.p.m.) loaded on to each gradient in parentheses were: at 10 min, ^{125}I 65% (232875), ^{131}I 75% (280325); at 30 min ^{125}I 60% (217050), ^{131}I 68% (112425). Some 70% of the homogenate *β*-*N*-acetylglucosaminidase activity was in the post-mitochondrial supernatant (77% recovered from gradient).

aminidase was: for leupeptin-treated rats, ^{125}I, 9±0.7% (3), ^{131}I, 29±4.1% (3); for a control rat, ^{125}I, 9% (1), ^{131}I, 10% (1).

The distributions of radioactivity from endocytosed ligands on both gradients were also examined by using livers from colchicine-treated rats (results not shown): under these conditions radiolabelled ASF remained with dense endosomes and was not transferred to lysosomes or degraded (cf. Kolset *et al.*, 1979; Berg *et al.*, 1985). pIgA transcytosis is inhibited by colchicine (Mullock *et al.*, 1980), and the gradient distribution of this ligand was not dissimilar to that in control experiments.

DISCUSSION

The data obtained from the present experiments suggest that ASF and pIgA are endocytosed into a common rat liver endosomal compartment, in agreement with previous electron-microscopic and subcellular-fractionation studies (Debanne *et al.*, 1984; Geuze *et al.*, 1984; Kindberg *et al.*, 1984; Courtoy *et al.*, 1985; Limet *et al.*, 1985). This compartment was identified as a light-endocytic-vesicle fraction by rapid isopycnic centrifugation on shallow Ficoll density gradients in a vertical rotor. Whereas the bulk of the liver pIgA remained associated with this hepatocyte compartment at later times after intravenous injection, ASF became increasingly associated with denser membrane fractions. Thus the light endocytic fraction appears to derive from the endosomal compartment known as CURL, the compartment of uncoupling of receptor and ligand, which is known to contain both ligands (Geuze *et al.*, 1984; Courtoy *et al.* 1985). The denser fractions probably correspond to the heavy-endosome fractions described by Limet *et al.* (1985) and to lysosomes. The densest peak on the Ficoll gradients contains much radioactivity, which centrifugation on Nycodenz gradients shows to be separable from classical lysosomes as defined by the presence of *β*-*N*-acetylglucosaminidase. This fraction may well correspond to the light-lysosomal fraction described by Berg *et al.* (1985) after fractionation on Nycodenz gradients. ASF also enters the classical lysosomal fraction, but its rapid degradation normally prevents much label being found in this region of Nycodenz gradients. Inhibition of this degradation with leupeptin to inhibit lysosomal proteinases (Dunn *et al.*, 1979) allowed us to show the co-localization of endocytosed ^{131}I-ASF radioactivity with *β*-*N*-acetylglucosaminidase on Nycodenz gradients. Leupeptin increased the density at which this marker was found on the gradients (results not shown), in agreement with previous studies (Dunn *et al.*, 1979). Leupeptin had no detectable effect on the distribution of endocytosed ^{125}I-pIgA on the gradients.

The apparent co-localization of latent 5′-nucleotidase activity with both light and dense endosome fractions on the Ficoll gradient is consistent with what might be expected for an internalized ectoenzyme (Stanley *et al.*, 1980; Luzio & Stanley, 1983). It has been reported that not all endocytic-vesicle fractions isolated from rat liver contain 5′-nucleotidase (Evans & Flint, 1985), but it does appear to be present throughout the endocytic pathways analysed in the present experiments, and latent activity is a useful additional marker of membrane fractions derived from them.

The present data demonstrate the usefulness of isopycnic centrifugation on a shallow Ficoll gradient in a vertical rotor in rapidly separating and identifying endocytic-vesicle compartments from rat liver. With a 1 h centrifugation under these conditions, sinusoidal plasma membrane, the light endocytic vesicles containing pIgA and the heavier endocytic vesicles into which ASF moves can all be distinguished from each other and from lysosomes. The method is therefore very suitable for the comparison of the subcellular distributions of different ligands and their variation with time after administration, and also for the examination of effects of inhibitors on endocytic pathways.

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