The time-dependent distribution of ^{125}I -asialo-orosomucoid–horseradish peroxidase and ^{131}I -immunoglobulin A among three endosomal subfractions isolated from rat liver

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Three discrete endosomal fractions showing a time-dependent uptake of radioactive ligand were partially purified from rat liver. The 3,3'-diaminobenzidine (DAB)-induced density-shift protocol of Courtoy, Quintart & Baudhuin [(1984) *J. Cell Biol.* **98**, 870–876] was used to study the distribution among these three endosomal fractions of two ligands with different intracellular destinations. Rats received both ^{125}I -asialo-orosomucoid–horseradish peroxidase (^{125}I -ASOR–HRP) and ^{131}I -dIgA simultaneously by intraportal injection. The liver was fractionated at various times after injection, the three ligand-containing endosomal fractions (A, B and C) were separated and each was subjected separately to the DAB-induced density-shift procedure in which only vesicles containing ^{125}I -ASOR–HRP are increased in density. Information on whether ^{131}I -dIgA was co-localized or segregated from ^{125}I -ASOR–HRP was obtained. The two ligands in the A fraction were partly segregated and partly co-localized, and this distribution appeared to be relatively unchanged with time. The two ligands in the B fraction were co-localized at all times studied. We have tentatively identified the B fraction as a compartment in which vesicle fusion has occurred. The two ligands in the C fraction were also partly co-localized and partly segregated, but the ^{131}I -dIgA became increasingly segregated with time. This represents the first report of the purification of an endosomal subfraction specifically involved in the accumulation of multiple ligands.

INTRODUCTION

Circulating asialoglycoproteins bind to specific receptors on the hepatocyte surface, are internalized through coated pits and delivered to lysosomes for ultimate degradation. Circulating IgA, in rats, also binds to a specific hepatocyte surface receptor, is internalized through coated pits, but by-passes the lysosomes and instead is directed to the bile-canalicular surface and discharged into bile. There is good evidence that both asialoglycoprotein and IgA, and their corresponding receptors, can occupy the same coated pit and be internalized together into the same compartment. An electron-microscopic study using antibodies to each of these receptors and ligands showed that, in rat liver, both of these receptors and their ligands were found together in coated pits and coated vesicles at frequencies of 51 and 37% respectively (Geuze *et al.*, 1984). This poses the problem of how molecules destined for different parts of the cell are sorted after being internalized together.

The two main ways in which this question has been studied are by electron microscopy and subcellular fractionation. Microscopy has provided useful information regarding the morphology of various endosomal compartments and the rate of transit of ligands and receptors through some of these structures. Subcellular fractionation has led to the identification of endosomal subcompartments differing in the presence or absence of specific receptors or ligands. Several endocytic structures

have been identified from microscopic studies. An initial uptake compartment where ligand is found immediately after internalization has been identified as coated vesicles. Coated vesicles have been isolated and shown to contain receptors for asialoglycoproteins (Steer *et al.*, 1983), mannose 6-phosphate (Campbell *et al.*, 1983) and low-density lipoprotein (Mello *et al.*, 1980). Ligand appears to move from coated vesicles to an endocytic compartment where various types of changes are induced to occur in the low-pH environment (Schwartz *et al.*, 1985) of this endosomal subcompartment. Examples of such changes are (i) separation of ligands from their receptors (Geuze *et al.*, 1983), and (ii) collecting certain receptors for recycling to the cell surface and separating them from receptors that are undergoing transcytosis together with their ligand, e.g. IgA (Geuze *et al.*, 1984). The information accumulated regarding the movements of receptors and ligands during endocytosis has not yet been matched by the isolation of organelles responsible for the various stages of the process.

There have been numerous reports concerning the isolation and purification of endosomes, but the relationship between these isolated structures and the functional compartments described from microscopic studies remains obscure. Evans & Flint (1985) and Mueller & Hubbard (1986) described the partial purification of endosomal compartments that differed in receptor composition. We have isolated three partially purified endosomal fractions (A, B and C) from rat liver that

Abbreviations used: DAB, 3,3'-diaminobenzidine; ^{125}I -ASOR–HRP, ^{125}I -asialo-orosomucoid–horseradish peroxidase; dIgA, human dimeric IgA.

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differ in their densities on Percoll gradients. We have used the 3,3'-diaminobenzidine (DAB)-induced density-shift procedure of Courtoy *et al.* (1984) with each of these three endosome fractions to try to discover whether they differ functionally. Two ligands labelled with different radioisotopes and having different destinations in the cell (^{125}I -ASOR-HRP and ^{131}I -IgA) were injected simultaneously in the portal vein of rats. Only endosomal fractions containing ^{125}I -ASOR-HRP can increase in density after incubation with DAB plus H_2O_2 .

Our results show that the three endosomal fractions differ in their DAB-induced density-shift profiles. The ^{131}I -IgA in the A fraction was partly segregated from the ^{125}I -ASOR-HRP. This distribution did not appear to change significantly with time. The two ligands in the B fraction were co-localized at all times studied. The two ligands in the C fraction were also partly co-localized and partly segregated, but the ^{131}I -IgA became increasingly segregated with time.

EXPERIMENTAL

Materials

Reagents were obtained from the following sources: Na^{125}I , [2- ^3H]AMP, UDP-D-[6- ^3H]galactose and [U- ^{14}C]glucose 6-phosphate were from Amersham Corp. (Arlington Heights, IL, U.S.A.); Percoll was from Pharmacia (Piscataway, NJ, U.S.A.); Formula-963 scintillation fluid was from New England Nuclear (Boston, MA, U.S.A.); male Sprague-Dawley rats were from Zivic-Miller (Allison Park, PA, U.S.A.); leupeptin, aprotinin, human α_1 -acid glycoprotein, CDP-choline, crystalline bovine serum albumin and horseradish peroxidase (HRP) (type VI) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); BCA protein assay reagent was from Pierce Chemical Co. (Rockford, IL, U.S.A.). Purified mouse IgA (TEPC 15) was from Bionetics Laboratory (Charleston, SC, U.S.A.) and was found to be dimeric by use of reducing and non-reducing gels. Human dimeric IgA (dIgA) was purified from myeloma serum by $(\text{NH}_4)_2\text{SO}_4$ fractionation (Heide & Schwick, 1978) and separation by h.p.l.c. on a Superose 6 column.

Asialo-orosomucoid (ASOR) was prepared by hydrolysis of α_1 -acid glycoprotein in 0.05 M- H_2SO_4 at 80 °C for 1 h. The solution was neutralized with solid NaHCO_3 and dialysed overnight against water. The removal of sialic acid was monitored as described by Warren (1959) and was found to be about 95% complete. Proteins (10 μg) were iodinated by the Iodogen procedure (Salacinski *et al.*, 1981) and purified by chromatography on Sephadex G-50 (medium grade; column dimensions 0.9 cm $\times$ 28 cm) equilibrated with 0.13 M- NaCl /0.12 M- NaH_2PO_4 (pH 7.3) 0.1% (w/v) bovine serum albumin. The peak fractions were pooled, divided into portions containing 15–45 μCi , and stored at -80°C for a maximum of 2 weeks. The specific radioactivity of the final product was 35–68 $\mu\text{Ci}/\mu\text{g}$. The portions were diluted to 400 μl with 0.15 M- NaCl before use.

Isolation of endosomal fractions

Rats weighing 225–300 g were fasted for 18–24 h. They were anaesthetized with pentobarbital administered intraperitoneally (50 mg/kg). The ^{125}I -ASOR was injected into the portal vein. At a given time after injection the liver was perfused via the portal vein with 100 ml

of cold 250 mM-sucrose/3 mM-Mops (pH 7.2)/10 mM-EDTA to remove surface-bound ligand. The liver was removed and homogenized in a Potter-Elvehjem homogenizer by using eight strokes of a loose-fitting (clearance 0.2 mm) Teflon pestle driven by a constant torque motor at 300 rev./min. The liver was homogenized in 25 ml of cold 250 mM-sucrose/3 mM-Mops (pH 7.2)/1 mM-EDTA/leupeptin (5 $\mu\text{g}/\text{ml}$)/aprotinin (5 $\mu\text{g}/\text{ml}$) (SME) and diluted with 45 ml of SME. The desired fractions were obtained by centrifugation at 4 °C in a Sorvall RC2B centrifuge using an SS-34 rotor (r_{av} 7.0 cm). The diluted homogenate was centrifuged for 10 min at 750 g , and the pellet was discarded. A 20 ml portion of SME was added to the postnuclear supernatant, which was then centrifuged for 5 min at 11 000 g and the pellet discarded. The 11 000 g supernatant was centrifuged for 15 min at 16 000 g and the pellet again discarded. The 16 000 g supernatant was re-centrifuged for 15 min at 22 000 g , the pellet discarded and the supernatant diluted to 78 ml with SME. The diluted 22 000 g supernatant was further purified by centrifugation in a 70 Ti rotor (r_{av} 6.57 cm) in an L8-70 centrifuge at 4 °C. The first stage was centrifugation for 35 min at 50 000 g . The resulting 50 000 g pellet was homogenized and adjusted to 6.0 ml with SME, whereas the 50 000 g supernatant was centrifuged for 35 min at 150 000 g . The resulting 150 000 g supernatant was discarded, whereas the pellet was homogenized and made up to 3.0 ml with SME.

The Percoll gradient solution was prepared by mixing 15.0 g of Percoll, 1.58 ml of 2.5 M-sucrose and 78 ml of SME and the solution divided equally among three tubes [1 in $\times$ 3.5 in (~ 2.5 cm $\times$ 9 cm)]. The 50 000 g pellet was layered in equal amounts on top of two of the tubes, whereas the 150 000 g pellet was layered on to the top of the third tube. The tubes were centrifuged for 30 min at 45 500 g in a 70 Ti rotor. Each gradient was then fractionated on an ISCO model 640 gradient fractionator,

Scheme 1. Fractionation procedure

80 0.4 ml fractions being collected. The fractions were collected from the top of the tube by pumping in a 'chase' solution of 60 % (w/v) sucrose. The radioactivity of each fraction was determined in an LKB 1272 Clinigamma counter. Samples containing both ^{125}I and ^{131}I were measured with a dual-label program.

The fractionation is summarized in Scheme 1. The procedure may be abbreviated, at some sacrifice of purity, by centrifuging the postnuclear fraction at 22000 *g* and eliminating the intervening steps. Washing of the 22000 *g* pellet markedly improves the ultimate recovery of injected ligand.

The vesicular components of peaks A, B and C could be isolated for further analysis by centrifugation for 1 h at 150000 *g*. The sedimented membranous material formed a sheet on top of a clear pellet of Percoll. The supernatant was discarded and the membrane sheet dislodged by addition of 200–400 μl of SME. The suspension was homogenized by hand in a 2 ml-volume homogenizer and could be kept at 4 °C for several weeks without clumping provided that the medium contains 0.5 mM-dithioerythritol and 0.1 mM-butylated hydroxytoluene. The suspension may also be stored at –80 °C without rupturing provided that the suspension is quick-frozen in solid CO_2 and quick-thawed by incubating 1 min at 37 °C.

Determination of density of Percoll gradient fractions

The procedure used was a modification of that described by Wolff (1975). A density-gradient column was made with four paraffin (kerosene)/chloroform mixtures, namely 94:31, 88:37, 81:44 and 75:50 (3:2) (v/v). After an overnight equilibration period the column was calibrated with sucrose standards of known densities. Samples (10 μl) were released just above the surface of the column.

Assay procedures

General features of enzyme assays 1–4 below. All assays were carried out at 37 °C, and assays 1–3 were terminated by heating at 95 °C for 2 min. The controls received water instead of a protein-containing sample. The reaction product was separated by ion-exchange chromatography on a column (0.9 cm $\times$ 3.0 cm) of Bio-Rad AG 1-2X-Cl resin (200–400 mesh). A 1 ml portion of water was added to the reaction medium after heating, and the contents were transferred to the column. The eluate was collected directly into 20 ml liquid-scintillation vials. The reaction tubes were rinsed (three times with 1 ml of water), and the rinsing were also put through the columns and collected in the same vials. The scintillation vials were changed and the columns washed four times with 1 ml of water. Each liquid-scintillation vial received 2.0 ml of 95 % (v/v) ethanol and 9.0 ml of liquid-scintillation fluid. All samples were counted for radioactivity (d.p.m.) in a Beckman 9000 liquid-scintillation counter. All assays were performed in duplicate and were linear with regard to incubation time (5–75 min) and protein concentration (10–150 μg) over the periods tested.

1. Glucose-6-phosphatase. The assay medium contained 50 mM-Mes, pH 6.6, 10 mM-unlabelled glucose 6-phosphate, 0.03 μCi of $[\text{U-}^{14}\text{C}]$ glucose 6-phosphate, 40 μg of protein or 25 μl of Percoll-gradient fraction plus water, in a final volume of 50 μl . Assay mixtures containing tissue or Percoll fractions were incubated for 10 or

20 min respectively at 37 °C. The addition of detergent to the assay medium was found to have no effect on the results.

2. 5'-Nucleotidase. The assay medium contained 50 mM-glycine/HCl, pH 9.0, 10 mM-MgCl₂, 0.25 % Triton X-100, 10 mM-unlabelled AMP, 0.1 μCi of $[\text{2-}^3\text{H}]$ -AMP, 80 μg of protein or 25 μl of Percoll-gradient fraction plus water, in a final volume of 50 μl . Assay mixtures containing tissue or Percoll fractions were incubated for 15 or 30 min respectively at 37 °C. Each assay tube received 5 μl of 0.5 M-sodium acetate, pH 4.5, immediately before ion-exchange chromatography.

3. Acidic 5'-nucleotidase. The assay medium contained 50 mM-sodium acetate, pH 4.5, 10 mM-MgCl₂, 0.25 % Triton X-100, 10 mM unlabelled AMP, 0.1 μCi of $[\text{2-}^3\text{H}]$ AMP, 80 μg of protein or 25 μl of Percoll-gradient fraction plus water, in a final volume of 50 μl . Assay mixtures containing tissue or Percoll fractions were incubated for 15 or 30 min respectively at 37 °C.

4. Galactosyltransferase. The assay medium contained 50 mM-Mops, pH 7.5, 30 mM-MnCl₂, 0.25 % Triton X-100, 25 mM-N-acetyl-D-glucosamine, 1 mM-dithioerythritol, 20 mM-CDP-choline (Senn *et al.*, 1981), 5 mM-UDP-galactose, 0.1 μCi of UDP-D- $[\text{6-}^3\text{H}]$ galactose, 20–300 μg of protein or 30 μl of Percoll-gradient fractions plus water in a final volume of 50 μl . All samples were incubated for 1 h at 37 °C and the reaction terminated with 25 μl of 0.3 M-EDTA, pH 7.4. Controls for this assay contained water in place of protein-containing solutions or had EDTA added before the addition of protein-containing solutions.

5. α -D-Mannosidase. The assay used was a modification of that described by Tulsiani *et al.* (1977). The assay medium contained 100 mM-sodium acetate, pH 5.5, 5 mM-p-nitrophenyl α -D-mannopyranoside, 0.25 % Triton X-100, 40 μg of protein and water, to a final volume of 500 μl . Samples were incubated for 20 min at 37 °C, and the reaction was terminated by the addition of 1000 μl of stopping solution (130 mM-glycine/67 mM-NaCl/85 mM-Na₂CO₃, pH 10.7). Absorbance was measured at 400 nm and corrected for a control containing water in place of protein.

6. β -Glucocerebrosidase. The assay used was a modification of the procedure of Aerts *et al.* (1986). The assay medium contained 85 mM-sodium citrate, pH 5.9, 0.12 % Triton X-100, 0.1 % sodium taurocholate, 5 mM-4-methylumbelliferyl β -D-glucopyranoside, 40 μg of protein and water, in a final volume of 230 μl . Samples were incubated for 1 h at 37 °C, and the reaction was stopped by addition of 800 μl of 100 mM-glycine, pH 9.1. The control contained water instead of protein. The product was measured in an Aminco-Bowman spectrofluorimeter by using 340 nm for excitation and determining emission at 448 nm.

7. Protein. This parameter was measured with BCA protein reagent using bovine serum albumin as the standard ($A_{280}^{1\text{cm}}$) and by heating the samples at 60 °C for 45 min. The absorbance was measured at 562 nm after cooling. Samples were solubilized by addition of Triton X-100 to a final concentration of 0.7 %.

Samples from Percoll gradients were first treated to remove Percoll, which interferes with the assay. The sample (50 μ l) was preincubated with 1% Nonidet P40 (35 μ l) for 10 min. Water (223 μ l) was added, followed by saturated $(\text{NH}_4)_2\text{SO}_4$, pH 6.6 (42 μ l), to give a final $(\text{NH}_4)_2\text{SO}_4$ concentration of 12% saturation. This caused the complete precipitation of the Percoll and no loss of protein. Tissue samples and 15 different purified proteins were tested for loss with this concentration of $(\text{NH}_4)_2\text{SO}_4$: none was detected. The addition of 225 μ l of undiluted Percoll in place of the water caused no interference with the determination of the protein standard or tissue-sample protein. After 20 min at 0 °C the samples were centrifuged in a Microfuge for 5 min at 4 °C to remove the precipitated Percoll. A sample (270 μ l) was removed, and 30 μ l of 1 M-Tris, pH 7.5, and 60 μ l of 60% (w/v) trichloroacetic acid were added, followed by 1.0 ml of 6% trichloroacetic acid. After 10 min at 0 °C the samples were sedimented for 20 min at 22000 g. Most of the supernatant (1.29 ml) was removed carefully and 1.5 ml of BCA reagent added to each tube. The samples were incubated at 60 °C for 45 min and the absorbance measured at 562 nm after cooling.

Preparation of ^{125}I -ASOR and ^{125}I -ASOR-HRP conjugate

The conjugate ^{125}I -ASOR-HRP was prepared and was purified on Sephadex G-150 as described by Nakane & Kawaoi (1974) by using 5 mg of unlabelled ASOR plus ^{125}I -ASOR (about 1 mCi) as radioactive tracer in place of IgG. The ASOR-HRP is cleared rapidly by hepatocytes through binding to the asialoglycoprotein receptor via the ASOR moiety of the conjugate (Wall *et al.*, 1980). The conjugate was electrophoresed on 5–17% (w/v) polyacrylamide gels and showed an M_r of 82000, the

anticipated value for a 1:1 complex. Each injection solution contained approx. 800 μ g of conjugate and was stored at –80 °C for a maximum of 2 weeks.

Preparation of ^{125}I -IgA

The dimeric IgA was iodinated (Salacinski *et al.*, 1981) by using 20 μ g of IgA and 10 mCi of Na^{131}I (500 mCi/ml; Amersham Corp. When dIgA was iodinated with Na^{125}I , the procedure was identical with that used for the iodination of ASOR. The iodinated dIgA was quick-frozen in solid CO_2 and stored, as samples containing 1–2 μ g of protein, at –80 °C for a maximum of 2 weeks.

Double-label injections

Male Sprague–Dawley rats, weighing 200–300 g, were fasted for 18–24 h and anaesthetized with an intra-peritoneal injection of pentobarbital (50 μ g/g body wt.). The ^{125}I -ASOR-HRP and ^{131}I -dIgA solutions were thawed by incubation at 37 °C for 1 min, mixed, and the mixture, containing 800 μ g of conjugate and 1–2 μ g of ^{131}I -dIgA, was injected into the portal vein. At the end of the desired time after injection, the needle was withdrawn and a cannula was inserted quickly into the portal vein. Cold SME, containing an additional 10 mM-EDTA, pH 7.4, was perfused through the liver and the liver excised and placed in cold SME buffer. The liver was then subjected to subcellular fractionation.

DAB-induced density shift

The procedure used was based on the method of Courtoy *et al.* (1984) and was modified as follows. A 6 mg/ml solution of DAB tetrahydrochloride (Polysciences, Warrington, PA, U.S.A.) in 0.25 M-sucrose/50 mM-imidazole buffer, pH 7.4, was prepared immediately before use. The pH of the DAB solution was adjusted to 7.4 with 5 M-NaOH and it was passed through 0.45 μ m-pore-size filters. Four of the desired peak fractions (approx. 1.5 ml) from the Percoll gradients were pooled and mixed with 1.5 ml of the DAB solution. A 50 μ l portion of 6% (w/v) H_2O_2 was added, and the reaction allowed to proceed in the dark at room temperature for 30 min with periodic mixing by inversion. Controls contained DAB, but H_2O_2 was omitted. The samples were then cooled, the entire solution layered over a 30 ml linear gradient of 28–55% (w/v) sucrose/3 mM-imidazole, pH 7.4, and centrifuged at 4 °C in a SW 28 rotor (r_{av} 11.82 cm; Beckman Instruments, Palo Alto, CA, U.S.A.) at 22000 g for 15–18 h. The sucrose gradients were fractionated and radioactivity was determined.

Assessment of ligand shifting in double-label experiments

All radioactivity falling within the control density range was considered unshifted ligand. All ligand outside the control density range was considered shifted ligand. The percentage of shifted and unshifted ^{131}I -dIgA was determined from plots of ^{131}I radioactivity against fraction number. The low level of soluble baseline radioactivity (1–10%) present throughout the entire DAB-shifted gradient was removed, and total particulate radioactivity was determined by weighing the appropriate portion of the graph paper. The shifted and unshifted peaks were then weighed separately to determine the

Fig. 1. Distribution of radioactivity, protein and marker enzymes from the 50000 g pellet on a Percoll gradient

The 50000 and 150000 g pellets were prepared from a liver removed at 4 min after ligand injection and centrifuged on separate Percoll gradients as described in the Experimental section. The values for radioactivity (●), protein (○), glucose-6-phosphatase (△) and 5'-nucleotidase (▲) are represented by the ordinates on the left, whereas those for acidic 5'-nucleotidase (□) and galactosyltransferase (■) are represented by the ordinate on the right. The bottom of the gradient is at fraction 80.

Fig. 2. Distribution of radioactivity, protein and marker enzymes from the 150000 g pellet on a Percoll gradient

The values for radioactivity (●), glucose-6-phosphatase (△) and 5'-nucleotidase (▲) are represented by the ordinates on the left, whereas protein (○) and density (◇) are represented by the ordinates on the right. The bottom of the gradient is at fraction 80.

amount of shifted and unshifted ^{131}I -IgA as a percentage of the total. In all experiments shown, 100% of the ^{125}I radioactivity shifted in the presence of DAB and H_2O_2 , and none of the ^{125}I radioactivity shifted in the control experiments.

Liver-perfusion studies

The isolated liver-perfusion system of Dunn *et al.* (1983) was used to assess the binding of ^{125}I -human dIgA to the rat asialoglycoprotein receptor. Ligand was allowed to bind to perfused rat liver for 1 h at 4 °C, unbound ligand was rinsed away, and the amount of ^{125}I -human dIgA released by perfusing the liver with 500 ml of buffer containing 10 mM-EDTA was taken as a measure of the amount of human dIgA bound to the asialoglycoprotein receptor. In experiments where an excess of unlabelled ASOR was used as competitor for the asialoglycoprotein receptor, either 400 or 800 µg of ASOR was mixed with the ^{125}I -human dIgA before injection into the perfusion system.

Electron microscopy

The DAB-shifted and control fractions were fixed for 30 min at 25 °C in Karnovsky's (1965) solution that was 0.5 osm instead of 2.01 osm and washed with 0.2 M-sodium cacodylate buffer, pH 7.3. The samples were post-fixed for 30 min at 25 °C in 1% OsO_4 /0.1 M-sodium cacodylate buffer, pH 7.3, washed extensively with water, fixed overnight at 4 °C with 1% tannic acid and then dehydrated in a series of graded ethanol solutions. The samples were embedded in Spurr plastic and thin sections were examined unstained in a JEOL 100 keV electron microscope.

RESULTS

Separation of three radioactive fractions

Figs. 1 and 2 show the results obtained when a liver was removed 4 min after injection of ^{125}I -ASOR and then fractionated as described in the Experimental section. The 50000 and 150000 g pellets were then centrifuged separately on Percoll gradients. Three peaks of radioactivity were seen. The 50000 g pellet generated two peaks on Percoll gradients. The low-density peak will be referred to as 'A' and the denser peak as 'B'. The 150000 g pellet showed a single peak (called 'C') with a tail. The variability of the positions of peaks A, B and C on the Percoll gradient was as follows: A, fraction 26 ± 4 ; B, fraction 61 ± 3 ; and C, fraction 20 ± 3 (mean $\pm$ s.d., $n = 70$). The merit of this procedure is that the shallow Percoll permits the separation of three distinct endosomal fractions, of very similar densities, which can be studied separately by use of the density-perturbation procedure.

Controls to rule out isolation of ligand-plasma-membrane complex

Several types of control experiments were done to be certain that the peaks of radioactivity observed on the Percoll gradients represented ligand in endocytic organelles rather than ligand bound to surface receptors in vesicles derived from plasma membrane. The addition of radioactive ligand to the homogenization medium containing a liver from an uninjected rat gave no peaks. Perfusion of the liver with 10 mM-EDTA or 100 mM-*N*-acetylgalactosamine after injection and before homogenization had no detectable effect. Both of these agents block ASOR binding and cause the release of ASOR already bound to its receptor (Harford *et al.*, 1984). The

Table 1. Recoveries in tissue fractions

The initial specific activities (nmol/min per mg of protein) in the postnuclear supernatant were: 5'-nucleotidase 30 ± 4 ; acidic-5'-nucleotidase, 14 ± 2 ; glucose-6-phosphatase, 99 ± 10 ; and galactosyltransferase, 0.8 ± 0.1 .

Parameter	n	Tissue fraction...	Recovery from postnuclear supernatant (%) (mean $\pm$ S.D.)						Total
			11 000 g pellet	16 000 g pellet	22 000 g pellet	50 000 g pellet	150 000 g pellet	150 000 g supernatant	
Radioactivity	22								
Protein	22								
5'-Nucleotidase	6		8 $\pm$ 1	10 $\pm$ 1	12 $\pm$ 1	48 $\pm$ 2	9 $\pm$ 1	9 $\pm$ 1	96 $\pm$ 1
Acidic 5'-nucleotidase	6		29 $\pm$ 2	5 $\pm$ 2	3 $\pm$ 1	10 $\pm$ 2	46 $\pm$ 7	46 $\pm$ 7	96 $\pm$ 5
β -Glucocerebrosidase	1		28 $\pm$ 3	13 $\pm$ 4	11 $\pm$ 4	24 $\pm$ 3	3 $\pm$ 1	3 $\pm$ 1	86 $\pm$ 4
Glucose-6-phosphatase	6		57 $\pm$ 3	13 $\pm$ 4	7 $\pm$ 1	9 $\pm$ 2	8 $\pm$ 4	8 $\pm$ 4	95 $\pm$ 7
Galactosyltransferase	6		75	10	4	5	1	6	101
α -D-Mannosidase	1		30 $\pm$ 3	9 $\pm$ 4	9 $\pm$ 4	40 $\pm$ 8	1 $\pm$ 1	1 $\pm$ 1	94 $\pm$ 6
	6		21 $\pm$ 5	9 $\pm$ 1	6 $\pm$ 1	11 $\pm$ 5	52 $\pm$ 9*	52 $\pm$ 9*	102 $\pm$ 8
	1		44	6	3	9	29	29	93

* Of the enzyme activity in this fraction, 85% was particulate (see the text for details).

implication of these results is that bound ASOR is internalized so rapidly that very little is present on the plasma membrane at any time. Alternatively, the homogenization process may in itself cause the release of surface-bound ligand. We routinely perfused all livers with SME/10 mM-EDTA before homogenization to ensure that the ligand-containing vesicles we isolated originated intracellularly. These experiments rule out the possibility that one or more of the peaks represent ligand bound to surface receptors. The presence of 100 mM-N-acetylgalactosamine throughout the entire purification process was also without affect.

Further analysis of Percoll gradients

The distributions of marker enzymes and protein on the Percoll gradients were also measured and the results are shown in Figs. 1 and 2. Neither the marker enzymes assayed nor protein peaks corresponded in position to the peaks of radioactivity on the gradient derived from the 50 000 g pellet. In addition, the bulk of the acidic-5'-nucleotidase, a marker for lysosomal activity (Burnside & Schneider 1982), is well separated from the peak of radioactivity (Fig. 1). In the gradient derived from the 150 000 g pellet (Fig. 2), the marker enzymes acidic-5'-nucleotidase and galactosyltransferase were not detectable. The enrichments achieved from the post-nuclear supernatant to the A, B and C peaks were 52-, 84- and 33-fold respectively.

We will show below that all of the marker enzymes tested can be removed completely from each of the three peaks of radioactivity by altering selectively the density of the endosomal fractions. This indicates these marker enzymes are not integral components of the endosomal fractions we have prepared.

Analysis of fractions isolated before the Percoll gradient

The tissue fractions were analysed for recovery of protein, radioactivity and several marker enzymes. These data are shown in Table 1. Almost 60% of the ligand radioactivity present in the postnuclear supernatant was found in the 50 000 g-plus-150 000 g pellets. The results show that these pellets contain the principal elements of the early stages of the endocytic pathway. The enrichment of ligand in these pellets is time-dependent (see Fig. 4 below) and is improved by washing the 10 000–22 000 g pellets.

The relatively high recovery of Golgi marker enzymes galactosyltransferase and α -D-mannosidase in the 150 000 g supernatant fraction was surprising, since they are particulate enzymes. When the 150 000 g supernatant was centrifuged at 190 000 g for 16 h, these enzymic activities were sedimented, whereas the radioactivity was not.

In this procedure the bulk of the lysosomes was removed before the isolation of the pellets containing the endosomal fractions. Most of the lysosomes were found in the 10 000 g pellet, as evidenced by the distribution of acidic 5'-nucleotidase and β -glucocerebrosidase.

Time-dependent uptake of ligands into liver

The effect of time on the appearance of various injected ligands into the postnuclear supernatant liver is shown in Fig. 3. We compared the uptakes of 125 I-ASOR, 125 I-dIgA and 125 I-horseradish peroxidase. The last has been used as a marker for fluid-phase pinocytosis (Steinman *et al.*, 1983), although it could also

Fig. 3. Rate of appearance of injected ligand into the postnuclear supernatant

Approx. $1 \mu\text{g}$ of radioactive ligand was injected in each case. $\circ$, ASOR; $\triangle$, human dimeric IgA; $\blacktriangle$, mouse dimeric IgA; and $\bullet$, horseradish peroxidase. The points with error bars show the means $\pm$ S.D. The numbers of measurements in each case were: 30 s, 5; 1 min, 7; 90 s, 4; 2 min, 6; 3 min, 16; 4 min, 14; 5 min, 7. All other points represent individual experiments.

be taken up via the mannose receptor in cells lining the liver sinusoids (Hubbard *et al.*, 1979; Quintart *et al.*, 1983). The half-time for appearance in liver of ^{125}I -ASOR is about 1 min, which agrees with the half-time for its disappearance from blood of 1 min (Wall *et al.*, 1980). The horseradish peroxidase was found to distribute itself on Percoll gradients exactly the same as ASOR, but the rate of internalization was considerably lower. Data are also shown for the rate of appearance of injected human and mouse dimeric ^{125}I -IgA in the postnuclear supernatant. The human dIgA is taken up more rapidly than that of the mouse, and both are taken up at a significantly lower rate than is ASOR. Both types of dIgA were found to be distributed in three peaks on Percoll gradients, similarly to ASOR.

Time-dependent labelling of the A, B and C peaks

A series of experiments were conducted in which the liver was removed at various times after injection of ^{125}I -ASOR. The distribution of radioactivity on the Percoll gradients was measured and the incorporation into each peak determined. These results are shown in Fig. 4.

Each of the three peaks showed a clear time-dependent accumulation of ligand, but they differ with regard to the maximum ligand content achieved. Most of the ligand is found in the compartment defined by the A fraction. It is important to emphasize that the data presented in Fig. 4 do not permit conclusions to be drawn regarding the presence or absence of kinetic interrelationships among vesicles of types A, B and C. Such conclusions would require a detailed documented model. Important undefined variables are: (i) the relationship between the amount of asialoglycoprotein injected and the amount in blood; (ii) the non-pulsatile uptake of ASOR into liver

Fig. 4. Effect of time on ligand incorporation into the endosomal fractions

Livers were removed at various times after intraportal ligand injection of ^{125}I -ASOR and treated as described in the Experimental section to prepare the A, B and C fractions on Percoll gradients. The radioactivity data were plotted on graph paper and the curves resolved into their components with a du Pont model 310 curve resolver. The incorporation into each peak was then determined by cutting and weighing the graph paper. The number of measurements in each case were: 30 s, 5; 45 s, 3; 1 min, 5; 2 min, 6; 3 min, 16; 4 min, 12; 5 min, 7; 6 min, 1; 8 min, 1. Points with error bars show the mean $\pm$ S.E.M. Fractions: $\circ$, A; $\bullet$, B; $\triangle$, C.

(Fig. 3); (iii) the specific radioactivity of ligand in each fraction as a function of time; (iv) the possible existence of multiple connections (Evans & Flint, 1985) between the three endosomal compartments described; and (v) entry of ligand through coated, as well as uncoated, pits (Sandvig *et al.*, 1987).

There is a slow steady rise in the appearance of radioactivity in the lysosome-enriched fractions (Table 1), from 30 s to 10 min after injection. This is followed by a sharp rise in the radioactivity in these fractions between 10 and 12 min after injection (results not shown).

DAB-induced-density-shift experiments

^{125}I -ASOR-HRP was injected into rats alone (single-label experiments) or together with ^{131}I -dIgA (double-label experiments). At various times after injection, the three endosomal fractions were prepared and each subjected to a DAB-induced density shift. This treatment causes the accumulation of dense oxidized DAB polymers in vesicles containing ^{125}I -ASOR-HRP, resulting in a marked increase in density of these vesicles. If the

Fig. 5. Typical DAB-induced density shift of endosomal fraction A

Rats were injected intraportally with 800 μ g of 125 I-ASOR-HRP, and ligand was allowed to circulate for 6 min after its injection. The liver was removed, endosomes were prepared, and peak fractions from the Percoll gradient were pooled and treated with DAB $\pm$ H_2O_2 as described in the Experimental section. The DAB-treated fractions were layered over continuous sucrose gradients and centrifuged. Fractions (80×0.4 ml) were collected and 125 I radioactivity determined. ●, Fractions treated with DAB + H_2O_2 ; ○, controls in which H_2O_2 was omitted; △, density. Similar results were obtained with the B and C peaks.

131 I-dIgA is present in the same compartment as the 125 I-ASOR-HRP, the 131 I-dIgA will also shift to a higher density. If 131 I-dIgA is present alone, the compartment remains at the control density. This protocol was used to study the sorting of two ligands in the three different endosomal fractions we have prepared.

A typical single-label DAB-shift experiment performed on the A endosome fraction is shown in Fig. 5. The ligand used was 125 I-ASOR-HRP, and therefore all of the 125 I radioactivity is shifted to a greater density than that of the control. Similar results were obtained with DAB shifts performed on the other two endosome fractions B and C. The starting densities of the three endosome fractions were approx. 1.14 g/ml on sucrose gradients, and the density of the DAB-shifted endosomes on sucrose was about 1.26 g/ml.

Marker-enzyme profiles were produced for both control (results not shown) and DAB-shifted gradients to rule out a shift in density due to non-specific agglutination of the endosomes with other organelles (Figs. 6a and 6b). The marker enzymes 5'-nucleotidase (plasma membrane), glucose-6-phosphatase (endoplasmic reticulum), acidic 5'-nucleotidase (lysosomes) and galactosyltransferase (Golgi) remained at the control density, whereas the radioactivity shifted to a higher

Fig. 6. Typical marker-enzyme profiles of fractions from the DAB-shifted gradient (+ H_2O_2) in Fig. 5

(a): ○, 5'-Nucleotidase; ●, glucose-6-phosphatase. (b): ○, Acidic 5'-nucleotidase; ●, galactosyltransferase. Similar results were obtained with all control gradients and with gradients from the DAB-shifted B and C fractions (not shown). The bottom of the gradient is at fraction 80.

density. It was not possible to measure the enrichment of ligand in the DAB-shifted fractions because of the difficulty of determining the protein content in the presence of the DAB polymer (Courtoy *et al.*, 1984; Ajioka & Kaplan, 1987). The DAB polymerization has also been reported to interfere with the assay of some marker enzymes (Courtoy *et al.*, 1984; Ajioka & Kaplan, 1987). The effect of this procedure on the recovery of marker enzymes from our control gradients was determined. It was found that 5'-nucleotidase and galactosyltransferase were unaffected, whereas glucose-6-phos-

Fig. 7. Electron micrographs of DAB-shifted and unshifted A and B fractions

Panel A, DAB-shifted A fraction; panel B, A-fraction control (unshifted); panel C, DAB-shifted B fraction; panel D, B-fraction control (unshifted); panel E, DAB-shifted C fraction; panel F, C-fraction control (unshifted). Peak fractions from gradients containing DAB-shifted or unshifted vesicles were fixed and processed to reveal oxidized DAB as described in the Experimental section. All photographs are at the same magnification, the horizontal bar in panel F representing 500 nm.

phatase and acidic 5'-nucleotidase were decreased by 86 and 58 % respectively (results not shown).

Electron microscopy was performed on the A, B and C DAB-shifted fractions to reveal the DAB reaction product inside the endosomes (Fig. 7). Tubular elements are a significant feature of these samples, and, in general, the B fraction appears to be largest and the C fraction the smallest. Most of the structures are filled with DAB reaction product. Control fractions were also examined (Fig. 7, panels B, D and F) and, as expected, did not contain significant amounts of DAB reaction product, showing that both DAB and H_2O_2 must be present to generate the reaction product. The control for fraction B (Fig. 7, panel D) had more matrix material than the other controls, but was clearly different in electron opacity from the shifted fractions (Fig. 7, panels A, C and E). The structures seen in the electron micrographs of the isolated endosomal organelles are similar to those observed in endosomal structures seen in tissue sections (Wall *et al.*, 1980; Mueller & Hubbard, 1986) or endosomes purified by the DAB-shift procedure (Courttoy *et al.*, 1984) from livers exposed to a ligand conjugated to HRP, namely a mixture of vesicles and tubular units that vary in size between 100 and 500 nm.

Differences in ligand distribution among the three endosomal fractions

Differences were observed among the three endosomal fractions when both ^{125}I -ASOR-HRP and ^{131}I -dIgA were injected simultaneously and the three fractions subjected individually to the DAB-shift procedure (Fig. 8). The ^{131}I radioactivity present in the B fraction was shifted completely to a higher density at all times after injection. This demonstrates that ^{131}I -dIgA and ^{125}I -ASOR-HRP are always together in this compartment, although their proportions may vary (see Fig. 9). With the C fraction, part of the ^{131}I was shifted and part unshifted, but a striking observation was that the percentage of ^{131}I that was unshifted showed an almost linear increase with time.

Analysis of the A fraction in this manner generated data that indicated this fraction was composed of at least two subcompartments. The curve representing fraction A shown in Fig. 8 was obtained by subjecting pooled samples, from the peak and fractions on either side of the peak of the Percoll gradients, to the DAB-induced density shift. Much of the ^{131}I was unshifted (not in the same compartment with ^{125}I -ASOR-HRP), and this distribution showed a relatively small increase with time.

An examination of the Percoll gradients generating the A and B peaks, from experiments in which both ligands were injected together, showed that the distribution of ^{125}I and ^{131}I radioactivities differed consistently by 5–7 fractions. This variation corresponded to a density difference of only 0.0008–0.0018 g/ml (Fig. 9). The lack of correspondence of the two isotopes in the A peak was studied by subdividing the peak into two portions and examining each one separately. A 'light' shoulder (A_1), corresponding to less-dense fractions in which the ^{131}I was maximum, and a 'heavy' shoulder (A_2), corresponding to the maximum peak of ^{125}I radioactivity, were subjected to the DAB-induced-density-shift procedure. The results are shown in Table 2. Most of the ^{131}I -dIgA in the A_1 shoulder was unshifted, whereas in the A_2 shoulder most of it was shifted, indicating differences

Fig. 8. Effect of time on the distribution of ^{125}I -ASOR-HRP and ^{131}I -dIgA among fractions A, B and C

The experimental protocol for double-label experiments is described in the Experimental section. The percentage of ^{131}I -IgA unshifted (ordinate) represents the amount of that ligand that is *not* present together with ^{125}I -ASOR-HRP in the same compartment. Fractions: ○, A; ●, B; △, C. The numbers of individual experiments for each point on the curve describing fraction B were as follows: 1 min, 2; 2 min, 2; 3 min, 3; 6 min, 3; 8 min, 1; and 10 min, 2. The error bars on the curve for fraction C represent $\pm$ s.d. ($n = 3$).

within the A compartment. The potential significance of this heterogeneity is discussed below.

The B compartment was also tested in this manner, but the shoulders behaved identically and were 100 % shifted even though the peaks of ^{125}I -ASOR-HRP and ^{131}I -dIgA did not coincide in this area of the Percoll gradient either (Fig. 9). Shoulders were not tested on the C fraction because the ^{125}I and ^{131}I radioactivities always coincided exactly on the gradient.

In all of the experiments, the controls (added DAB but no H_2O_2) remained unshifted for both isotopes. In addition, 100 % of the ^{125}I radioactivity in the DAB + H_2O_2 gradients were shifted, for all three endosomal fractions, at all times studied (results not shown). This is a necessary condition to ensure that the HRP moiety is still attached to the ^{125}I -ASOR and that the reaction has gone to completion.

Assessment of binding of human dIgA to the rat asialoglycoprotein receptor

Jones *et al.* (1985) compared the uptake and metabolism of human and rat polymeric IgA by rat liver and concluded they were processed differently. Evidence that human polymeric IgA can bind to the rat asialoglycoprotein receptor as well as to the rat IgA receptor was

Table 2. Heterogeneity of fraction A

Rats received ^{125}I -ASOR-HRP plus ^{131}I -dIgA, and endosomal fractions A and B were prepared from livers removed at the indicated times after ligand injection. The shoulders (see the legend to Fig. 9) were allowed to react with $\text{DAB} \pm \text{H}_2\text{O}_2$, and the percentage of ^{131}I -dIgA remaining unshifted was determined as described in the Experimental section. Results are means $\pm$ S.D. (n).

Endosome fraction	Time (min) ...	Percentage of ^{131}I -dIgA unshifted		
		3	6	10
A ₁		72.2 $\pm$ 7.6 (3)	87.0	85.7
A ₂		28.5 $\pm$ 2.5 (3)	41.9	36.7

reported (Schiff *et al.*, 1986). The results obtained by these investigators could affect the interpretation of our double-label DAB experiments, since we used human dIgA as a probe for the transcellular pathway. We therefore addressed this question in the context of our own experiments. The finding cited above, namely that human dimeric IgA binds to rat asialoglycoprotein receptor, was tested directly in two ways. In the first, rat liver was perfused with ^{125}I -human dIgA at 4 °C and the total amount of bound ligand determined. The liver was then perfused with cold buffer containing 10 mM-EDTA to dissociate any binding to the asialoglycoprotein (Ca^{2+} -dependent) receptor. This treatment does not dissociate binding to the IgA receptor, which is not Ca^{2+} -dependent. Any ^{125}I -human dIgA bound to the asialoglycoprotein receptor should be released into the medium under these conditions. A second test was to determine whether the binding of human dIgA could be inhibited by having a large excess of ASOR present in the binding medium. The results are shown in Table 3. The first type of experiment showed that approx. 57% of the ^{125}I -human dIgA in the medium was bound, and less than 5% of the bound ligand was released by subsequent perfusion with 10 mM-EDTA. In contrast, 98% of bound ASOR was released by 10 mM-EDTA. The second type of experiment showed that the binding of dIgA was not affected significantly by the presence of a large excess of ASOR.

Fig. 9. Double-label experiment showing distribution of ^{125}I and ^{131}I radioactivity in fractions A and B

The experimental protocol for double-label experiments is described in the Experimental section. The liver was removed 4 min after injection of ligands. $\circ$, ^{125}I ; $\bullet$, ^{131}I . Fractions 19–22 were taken to test the A₁ shoulder and fractions 36–39 to test the A₂ shoulder; fractions 60–63 and 74–78 were used to test B shoulders. The bottom of the gradient is at fraction 80.

These results indicate that our human dIgA does not bind appreciably to the asialoglycoprotein receptor in the presence or absence of additional ASOR. These results support similar previous findings for cultured rat hepatocytes and the finding that transfer of human

Table 3. Binding of ^{125}I -human IgA and ASOR to isolated perfused rat liver and their release by 10 mM-EDTA

Rat livers were maintained in a 4 °C perfusion system with recirculating oxygenated Krebs–Ringer bicarbonate as described by Dunn *et al.* (1983). The ligand(s) were allowed to bind to the liver for 60 min. At the end of the binding period, unbound ligand was washed out and the liver was then perfused with 500 ml of Krebs–Ringer bicarbonate + 10 mM-EDTA to release ligand bound to the asialoglycoprotein receptor.

Ligand	ASOR* (μg)	Surface-bound radioactivity (%)	Bound radioactivity released by 10 mM-EDTA (%)
^{125}I -human IgA	0	56.9 $\pm$ 5.8†	3.6 $\pm$ 0.1†
	375	61.5	5.1
	800	52.5	1.9
^{125}I -ASOR	—	75.8 $\pm$ 5.5‡	98.3 $\pm$ 1.2‡

* ASOR was mixed with tracer quantities of ^{125}I -human IgA before addition to the perfusion medium.

† Mean $\pm$ S.D., $n = 3$.

‡ Mean $\pm$ S.D., $n = 17$; at the end of the ligand wash-out period, the liver was perfused with 500 ml of non-recirculatory Krebs–Ringer bicarbonate plus 10 mM-EDTA; the perfusate was collected and total radioactivity released was measured.

polymeric IgA to bile by rat liver was unaffected by simultaneous administration of large amounts of galactosylated bovine serum albumin (Courtroy *et al.*, 1985).

In addition, we have carried out DAB-induced-density-shift experiments using mouse dIgA instead of human dIgA and have obtained similar results in the double-label DAB-induced-density-shift experiments. Mouse dIgA is not galactosylated and therefore does not bind to the asialoglycoprotein receptor (Robinson & Appella, 1980).

DISCUSSION

Suitability of the DAB-induced-density-shift procedure for studying endosomes and the sorting process

The DAB-shift method has been shown to be useful in purifying organelles free of cellular contaminants (Courtroy *et al.*, 1984; Quintart *et al.*, 1984; Limet *et al.*, 1985). The procedure is highly selective in that it produces a density increase only in those organelles containing HRP. This makes it possible to test whether a ligand not conjugated to HRP is present in the same compartment with a second ligand that is. The DAB reaction is reproducible and, when coupled with the use of different radiolabels on two proteins with different destinations in the liver, can be used to study sorting in endosomes (Courtroy *et al.*, 1982, 1985; Limet *et al.*, 1985). We have described the partial purification of three distinct endosome fractions from Percoll gradients and have further purified each one separately using the DAB-shift procedure. We found these density-shifted organelles were essentially devoid of 5'-nucleotidase, glucose-6-phosphatase, acidic 5'-nucleotidase and galactosyl-transferase activities as judged by marker-enzyme analysis. These results are in agreement with those of Quintart *et al.* (1984).

Comparison with other studies

The original studies done with the DAB-shift method in the purification of endosomes from rat liver (Courtroy *et al.*, 1984; Quintart *et al.*, 1984) used a microsomal fraction obtained from sucrose gradients. We have used as our starting material three different endosomal fractions obtained from Percoll gradients. These three fractions have similar densities on sucrose or more concentrated Percoll gradients, but differ on the relatively dilute Percoll gradients we used. Courtroy *et al.* (1982, 1985) and Limet *et al.* (1985) studied the sorting of galactosylated bovine serum albumin and polymeric IgA in rat hepatocytes using the DAB-shift method. They found that the two ligands were internalized into the same compartment, but were sorted into separate structures within 10 min. This finding agrees with our own observations regarding the kinetics of sorting in rat liver endosomes. We have extended these findings by defining three fractions differing in the co-localization of both ASOR and dIgA as a function of time (Fig. 8).

Branch *et al.* (1987) also studied the segregation of an asialoglycoprotein, asialofetuin, and IgA by rat liver. They found that the two ligands were at about the same position on gradients initially, whereas by 5 min after injection a significant portion of the asialofetuin had moved to a position of greater density on the gradient, suggesting that some sorting had occurred. Stoerovogel *et al.* (1987) used the DAB-shift procedure to examine the sorting of ^{125}I -ASOR-HRP and ^{131}I -transferrin by

HepG2 cells. They found that the sorting of these two proteins had a half-time of about 2 min and was largely completed by 10 min. The preceding studies did not involve the isolation and examination of specific endosomal fractions or an assessment of the sorting ability of any of the gradient peaks.

Possible functional roles of the three endosomal fractions

The three endosomal fractions we have isolated (A, B and C) differ functionally (Fig. 8) and morphologically (Fig. 7). It is believed that ASOR and polymeric IgA can enter liver cells in the same or separate vesicles. Geuze *et al.* (1984) used immuno-electron microscopy to show that asialoglycoprotein and polymeric IgA surface receptors are co-localized in the same coated pits on the plasma membrane with a frequency of 35–40 %, but that they were also found separately in coated pits. This type of receptor distribution could be real or a reflection of the relatively low efficiency of immunolabelling. Both receptors were co-localized within the compartment termed 'CURL', but within distinct domains. 'CURL' is an endocytic compartment in which ligand and receptor are segregated, but may also be a compartment in which different ligands are segregated. Since the receptor for polymeric IgA was found alone in coated pits as well as co-localized with the asialoglycoprotein receptor, it is possible that polymeric IgA can enter the hepatocyte either alone or in the same compartment with asialoglycoprotein. According to these investigators, the distribution of these two receptors is entirely random at the sinusoidal hepatocyte surface. Vesicles generated directly from the plasma membrane would therefore be anticipated to be composed of a mixture of those containing both ligands and those containing one ligand or the other.

It appears that compartment A contains an assortment of ASOR and IgA molecules, some of which are co-localized and some of which are not. The DAB-shift profile of the A compartment shows that IgA is shifted along with the ASOR-HRP at a frequency of 32–48 %. This number is in agreement with the frequency of co-localization described above that was obtained by Geuze *et al.* (1984). This finding is consistent with the possibility that the A fraction may represent a compartment close to the plasma membrane. The A₁ shoulder is the subfraction enriched in IgA present alone and may represent a fraction generated in large part from coated pits containing only IgA receptors. This is not meant to imply that the sorting of these two ligands occurs primarily at the cell surface. The A₂ shoulder is the subfraction containing mainly IgA and ASOR-HRP in the same compartment and may have arisen primarily from coated pits containing both IgA and ASOR receptors. A partial contamination of the A₂ shoulder with fraction B cannot be excluded. The slight effect of time on the distribution of the two ligands in the A fraction, which peaks at about 6 min (see Fig. 8 and Table 2), may result from the difference in the rate of uptake into liver of ASOR and IgA (see Fig. 3).

The B fraction showed 100 % co-localization of both ligands from 1 to 10 min after ligand injection. This time range covers the major part of the endocytic process, since, by 12 min after injection, ligand has reached the lysosomes (see the subsection 'Time-dependent labelling of the A, B and C peaks' above). The most interesting

possibility is that the B fraction is a fusion compartment formed by the coalescence of vesicles derived from coated pits containing one or both ligands. The proportions of the two ligands present together in such a compartment would be variable and dependent on the frequency of colocalization, in a single coated pit, of receptors for the ligand we tested. The results shown in Fig. 9 indicate that the distribution of the two ligands on the gradient do not coincide for peak B and support the supposition that the two ligands are present in variable amounts in fraction B. In spite of this lack of coincidence, the two ligands always move together after the DAB-induced shift in density. This was true even when we examined the shoulders on the two sides of the peaks. The identification of fraction B as a fusion compartment is also supported by our finding that this fraction appears to have the largest size (Fig. 7). Electron-microscopic studies have shown that the 'CURL' apparatus, the compartment in which various ligands are found together, appears to have a central vesicle with a diameter of about 400–500 nm (Geuze *et al.*, 1983). An alternate explanation is that fraction B represents vesicles derived exclusively from coated pits containing both receptors and ligands in various proportions. These structures would have to have a very different density, and possibly size, from vesicles derived from coated pits containing mainly a single ligand. Such an explanation is more difficult to accept, since examination of tissue sections has shown that the earliest vesicles formed appear to about 100 nm in diameter (Wall *et al.*, 1980; Geuze *et al.*, 1983).

Finally, we have shown that a time-dependent change in the relative distribution of ^{125}I -ASOR-HRP and ^{131}I -dIgA occurs in the C fraction (Fig. 8). There is a progressive increase in the proportion of the IgA that is in a vesicle by itself and, at 10 min after injection, about 70% is in a compartment different from that of ASOR. These results are consistent with at least two different explanations. The first is that fraction C represents a compartment in which ligand sorting occurs. This possibility would have to account for the fact that this fraction contains ligands as soon as 30 s after injection. A second interpretation is that fraction C represents small vesicles newly generated from the plasma membrane and that the time-dependent changes shown in Fig. 8 reflect differences in the rates of clearance from blood of ^{125}I -ASOR-HRP and ^{131}I -dIgA.

It is worth re-emphasizing that the identification of our three fractions was dependent on the preparation procedure we have described and is not achieved in procedures utilizing gradients of sucrose or greater concentrations of Percoll.

This work was supported by National Institutes of Health grant GM 31566. A training grant from the National Institutes of Health (AM 07319) provided support for G.K. The data reported are taken from a thesis submitted by G.K. in partial fulfilment of the requirements for a Ph.D. degree of Case Western Reserve University. We express our appreciation to Mr. Nicholas Dalton for the technical help provided, Ms. Helga Beegan for the electron microscopy work, Dr. Charlotte Kaetzel for providing human dimeric IgA and Dr. Martin Snider and Dr. Alan Tartakoff for their suggestions regarding the writing of this manuscript.

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Received 8 October 1987/26 January 1988; accepted 22 February 1988