Receptor-mediated Endocytosis in Rat Liver: Purification and Enzymic Characterization of Low Density Organelles Involved in Uptake of Galactose-exposing Proteins

JOËL QUINTART, PIERRE J. COURTOY, and PIERRE BAUDHUIN
Laboratoire de Chimie Physiologique, University of Louvain and International Institute of Cellular and Molecular Pathology, B-1200 Brussels, Belgium

ABSTRACT Rat liver organelles involved in receptor-mediated endocytosis were labeled with a conjugate of galactosylated BSA to horseradish peroxidase ([^{3}H]galBSA-HRP), injected 10 min before sacrifice. These organelles were recovered at low density (1.11–1.13 g/ml) in sucrose gradients (Quintart, J., P. J. Courtoy, J. N. Limet, and P. Baudhuin, 1983, *Eur. J. Biochem.*, 131:105–112). Upon incubation of such low density fractions in 3,3′-diaminobenzidine (DAB) and H_2O_2 and equilibration in a second sucrose gradient, galBSA-HRP–containing particles selectively shifted towards heavier densities (Courtoy, P. J., J. Quintart, and P. Baudhuin, 1984, *J. Cell Biol.*, 98:870–876, companion paper), resulting in up to 250-to 300-fold purification with respect to the homogenate. The most purified preparations, wherein DAB-stained structures represented ~85% of the total volume of particles, contained only trace activities of enzymes usually regarded as markers for other subcellular entities. These minor activities could reflect either contamination or true enzyme association to the ligand-containing structures. Considering the latter hypothesis, at most 1.0% of alkaline phosphodiesterase I and 2.6% of 5′-nucleotidase (markers for plasma membrane), 3.6% of *N*-acetyl-β-glucosaminidase (lysosomes), and 6.0% of galactosyltransferase (Golgi complex) from the homogenate would be associated with the whole population of ligand-containing organelles. After DAB cytochemistry on liver fixed 10 min after galBSA-HRP injection, ligand-containing structures accounted for 0.78–0.89% of the fractional volume of the hepatocytes and displayed a membrane area of 2,100 cm^2/cm^3, compared with 6,700 cm^2/cm^3 for the pericellular membrane. Altogether, our data support the hypothesis that these ligand-containing organelles are structurally distinct from plasma membrane, lysosomes, and Golgi complex.

Rapidly after interiorization, numerous ligands taken up by receptor-mediated endocytosis (7, 31, 44) are found associated with structures equilibrating at low density (15, 21–23, 26, 30, 35, 40, 41, 48). Electron microscopy shows that ligands are concentrated in clathrin-coated pits or vesicles, and rapidly transferred into electron-lucent, smooth surfaced organelles (35, 45, 47). These structures have received several names including receptosomes (52), endosomes (16), endocytic vesicles (1), compartment of uncoupling of receptor and ligand, or CURL (13), and ligandosomes (41).

Using galactosylated BSA conjugated to horseradish peroxidase (gal BSA-HRP),[1] we have recently been able to identify in rat liver fractions numerous smooth vesicles or tubules labeled with HRP reaction product. These fractions were, however, still largely contaminated (35). We report here on the use of a 3,3′-diaminobenzidine (DAB)-induced density shift (8) for further purification of galBSA-HRP–containing structures. The contribution of ligand-containing structures

[1] *Abbreviations used in this paper:* DAB, 3,3′-diaminobenzidine; galBSA, galactosylated BSA; galBSA-HRP, galBSA conjugated to horseradish peroxidase (HRP); L_l pool, L-derived light pool; LP_l pool, LP-derived light pool; RSA, relative specific activity.

to liver protein has been estimated from the relative specific activity (RSA) of ligand in purified preparations and has been correlated to the fractional volume of these structures in intact liver, as evaluated by morphometry. Marker enzymes for other subcellular entities were found in trace amounts in the highly purified fractions. The significance of these observations will be discussed within the framework of current concepts on membrane recycling, subcellular compartment individuality, and sorting processes. Part of this work has been published in abstract form (33, 36).

MATERIALS AND METHODS

Protein Determination and Enzyme Assays: Since DAB interferes with the colorimetric protein determination of Lowry et al. (27), male Wistar rats (200–250 g) were injected intraperitoneally with a pulse of [^{14}C]leucine or [^{35}S]methionine (125–250 μCi), 40 h before sacrifice, so as to label liver protein. The percentage of [^{14}C] or [^{35}S] label, with respect to the homogenate, was used to assess the protein content of the subcellular fractions.

Activities of the following marker enzymes were determined: *N*-acetyl-β-glucosaminidase (24), 5′-nucleotidase (3), alkaline phophodiesterase I, galactosyltransferase, esterase, and glucose 6-phophatase (4).

Cell Fractionation: To label the organelles involved in the galactose specific pathway, rats were injected intravenously with 1 μg/g body wt of galactosylated BSA ([^{14}C]galBSA, 7 nCi/μg) or of its conjugate to horseradish peroxidase ([^{3}H]galBSA-HRP, 126 nCi/μg) prepared as described in the companion paper (8). 10 min after injection, the liver was perfused by the portal vein with tissue culture medium, removed, and homogenized in 3 vol of 0.25 M sucrose buffered with 3 mM imidazole/HCl, pH 7.

For differential centrifugation, the procedure described by de Duve et al. (9) was adopted with some modifications. The nuclear (N) fraction (10,000 g·min) and the heavy mitochondrial (M) fraction (33,000 g·min) were sedimented either separately or in a combined NM fraction. The N and NM pellets were resuspended in buffered sucrose, using a tissue grinder, model C (Arthur H. Thomas Co., Philadelphia, PA.). The supernatant of this last centrifugation was further fractionated in two ways. In a first procedure, the light mitochondrial (L) fraction (250,000 g·min) and the microsomal (P) fraction (3,000,000 g·min) were successively pelleted as described by de Duve et al. (9), except that the L fraction was washed once and the P fraction was not. In the second procedure, a combined LP fraction was directly obtained by omitting prior isolation of the L fraction. This LP fraction was not washed. The L, P, and LP pellets were resuspended using a Dounce homogenizer with a loose pestle (Kontes Glass Co., Vineland, NJ).

For isopycnic centrifugation, L or LP fractions were equilibrated in sucrose gradients as described previously (35). In a first series of experiments, fractions were collected by pumping the gradient starting from the bottom of the tube. The pools of low density fractions, denoted LP_1 or L_1 depending on the starting material, corresponded to fractions spanning the range of 1.11–1.13 g/ml in density. The biochemical composition of these preparations is illustrated in Fig. 1. Later, in order to minimize contamination of low density fractions by the organelles equilibrating at higher density, we collected the L_1 pool using a tube slicer adapted for 2.5-cm-diam tubes, from the device originally described by de Duve et al. (10).

A density shift of the galBSA-HRP–containing structures was then induced by incubation of either LP_1 or L_1 pools in the presence of 5.5 mM DAB and 11 mM H_2O_2, followed by reequilibration in a linear sucrose gradient as described in the companion paper (8). In some experiments, after the cytochemical procedure, the preparations were layered on a discontinuous sucrose gradient. The latter was made of three layers of 21, 11, and 2 ml with densities of 1.18, 1.24, and 1.34 g/ml, respectively.

Electron Microscopy: Subcellular fractions obtained after the DAB-induced density shift were processed as described previously (8) except that ~10 μg of protein was applied over 0.04 cm^2, 100-nm nominal pore size filter (Millipore Corp., Bedford, MA.).

On intact tissue, peroxidase cytochemistry (14) was performed on slices from glutaraldehyde-perfused liver as described previously (35). Briefly, after 20-min preincubation of liver slices in 0.05 M Tris buffer, pH 7.0, containing 1 mg/ml DAB/4 HCl, peroxidase cytochemistry was started by addition of H_2O_2 (0.01% final concentration) and allowed to develop for 30 min in the dark at room temperature. Ultrathin sections were examined without counterstaining. For stereological analysis, each section photograph was taken from a different ribbon, cut at a distance of at least 20 μm (more than the cellular diameter) in order to analyze different cells.

The magnification was determined by using a grating replica (E. F. Fullam Inc., Schenectady, NY). A multipurpose test grid similar to that described by Weibel et al. (49) was used for the determination of volume fraction or membrane area. For the recording and processing of morphometrical data, an Apple II plus (Apple Computer Inc., Cupertino, CA) was used.

RESULTS

Subfractionation of LP Fractions

When an LP_1 pool was incubated in the presence of DAB and H_2O_2 and submitted again to equilibration in sucrose gradient, the distribution of galBSA-HRP–containing structures was largely shifted (~0.06 g/ml) towards heavier density (Fig. 2). By contrast, the bulk of [^{14}C]leucine, *N*-acetyl-β-glucosaminidase (marker for lysosomes), galactosyltransferase (Golgi complex), and 5′-nucleotidase and alkaline phosphodiesterase I (plasma membrane) still equilibrated at low density. Their median equilibrium density was only slightly increased (~0.01 g/ml) when compared with controls incubated in the presence of DAB alone. The small amounts of glucose 6-phosphatase and esterase present in the pools did not allow reliable determinations for these enzymes.

After DAB-induced density shift, 13.2% of the ligand present in the homogenate, but <0.1% of the aforementioned marker enzymes, were recovered in the most enriched fraction (Table I). When compared with the LP_1 pool, the density equilibration after incubation in DAB and H_2O_2 resulted in a further sixfold purification of the ligand-HRP–containing

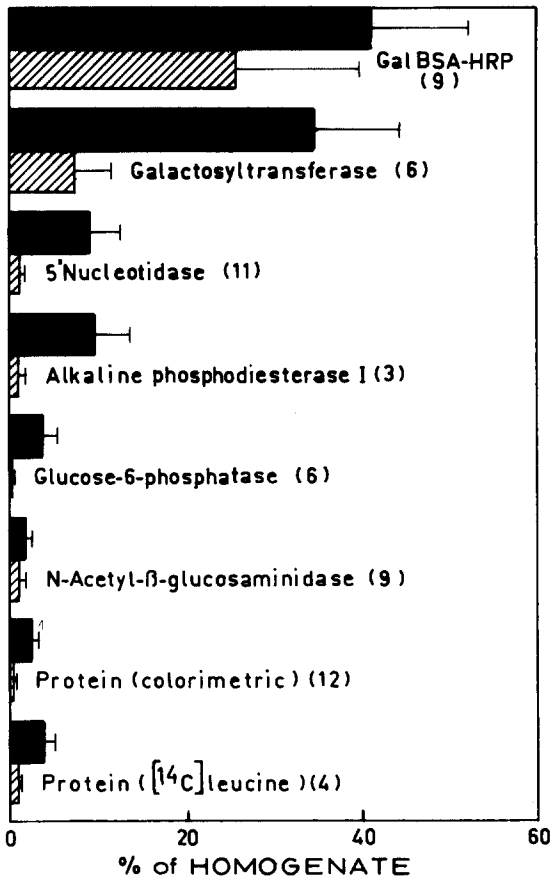

FIGURE 1 Composition of LP_1 and L_1 pools. Rats were injected with [^{14}C]leucine and [^{3}H]galBSA-HRP 40 h and 10 min, respectively, before sacrifice. LP_1 (solid rectangles) and L_1 pools (hatched rectangles) were prepared as described in Materials and Methods. Values are average percentages ± SD, with respect to the homogenate. Number of experiments is given in parentheses.

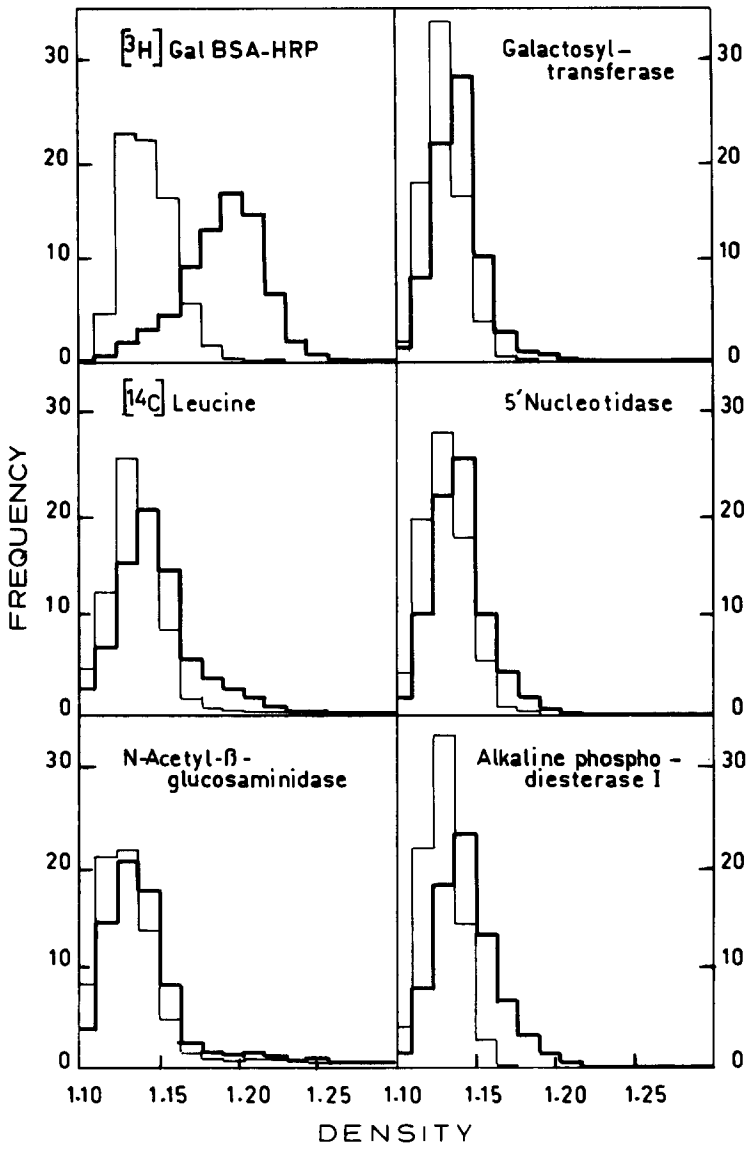

FIGURE 2 Density distributions after DAB-induced density shift of a LP_1 pool. Rats were injected with [^{14}C]leucine and [^{3}H]galBSA-HRP, 40 h and 10 min, respectively, before sacrifice. An LP_1 pool was isolated and incubated in 5.5 mM DAB in 0.74 M sucrose (density 1.1 g/ml) buffered with 3 mM imidazole/HCl pH 7, in the absence (thin lines) or presence (thick lines) of 11 mM H_2O_2. After 30-min incubation at 25°C in the dark, under gentle agitation, the preparation was equilibrated again on linear sucrose gradients. Density distributions were standardized according to Leighton et al. (25). With respect to the LP_1 pool, recoveries were between 77 and 119%. In this typical experiment, the RSA of galBSA-HRP (ratio of percentage of galBSA-HRP and that of [^{14}C]leucine) was in the LP_1 pool and in the most enriched fraction after the DAB-induced density shift, 11.3 and 89.5, respectively.

TABLE I
Composition of Shifted Material from LP_1 Pools

Marker	% of the homogenate	RSA
[^{14}C]leucine	0.157 ± 0.036	—
[^{3}H]galBSA-HRP	13.2 ± 2.0	84.0 ± 28.9
Galactosyltransferase	0.083 ± 0.013	0.54 ± 0.10
5'-Nucleotidase	0.059 ± 0.017	0.41 ± 0.21
Alkaline phosphodiesterase I	0.085 ± 0.078	0.49 ± 0.46
N-Acetyl-β-glucosaminidase	0.037 ± 0.029	0.21 ± 0.16

LP_1 pools from three different rats were incubated in DAB and H_2O_2 and equilibrated again in linear sucrose gradients. Values are means ± SD for the fraction with the highest galBSA-HRP specific activity in each experiment. These fractions were recovered between densities of 1.211 ± 0.013 and 1.225 ± 0.013 g/ml. [^{14}C]leucine incorporation was used to assess the protein content. RSA corresponds to the ratio between the percentage of marker enzyme and that of [^{14}C]leucine, with respect to the homogenate.

structures, based on [^{14}C]leucine-labeled protein content (Table II). The value of 84 evaluated for the final RSA of galBSA-HRP is most probably underestimated. Indeed, the ratio of the protein content measured by [^{14}C]leucine incorporation and that determined with the assay of Lowry et al. (27) was on average 1.18 in LP fractions and 1.68 in LP_1 pools.

Subfractionation of L Fractions

Since a higher RSA for galBSA-HRP–containing structures can be obtained in the low density pool isolated from an L rather than from an LP fraction (35, and Fig. 1), the DAB procedure was next applied to L_1 pools (Fig. 3). As can be seen, the shift of galBSA-HRP was similar to that observed with the LP_1 pool, but with the L_1 pool used here, ~45% of the [^{14}C]leucine-labeled protein migrated with the ligand. For 5'-nucleotidase, alkaline phophodiesterase I, and galactosyltransferase, the displacement of density distribution was more noticeable than with the LP_1-derived preparations presented in Fig. 2. The displacement was smallest for *N*-acetyl-β-glucosaminidase. Nevertheless, the peak of displaced [^{14}C]-leucine and enzymes did not coincide with the peak of the shifted ligand. Average data obtained on DAB-induced density shifts performed on L_1 pools are presented in Table III.

Comparison of the enzymic content of the preparations of Tables III and I shows that the higher value of RSA for galBSA-HRP–containing structures in the shifted material isolated from the L_1 pool did not correspond to a decrease in the amount of galactosyltransferase, 5'-nucleotidase, alkaline phophodiesterase I, and *N*-acetyl-β-glucosaminidase. Hence, the subcellular entities bearing these markers are not the

TABLE II

Subfractionation of LP Fractions

	Protein			
	Lowry et al. (27)	[^{14}C]leucine	[^{3}H]galBSA-HRP	RSA
	%	%	%	
LP fraction	29.4 ± 1.5	34.7 ± 2.6	81.9 ± 3.2	2.4 ± 0.2
LP_1 pool	2.5 ± 0.6	4.2 ± 1.2	56.8 ± 6.1	14.3 ± 4.0
Shifted material	—	0.16 ± 0.04	13.2 ± 2.0	84.0 ± 28.9

Protein, as determined by the procedure of Lowry et al. (27), or by [^{14}C]leucine incorporation, and [^{3}H]galBSA-HRP content are given for the three major steps of the purification procedure. Shifted material is defined in Table I. Percentages are expressed with respect to the homogenate. RSA corresponds to the ratio of the percentage of galBSA-HRP and that of [^{14}C]leucine in each fraction. Mean values ± SD for three experiments.

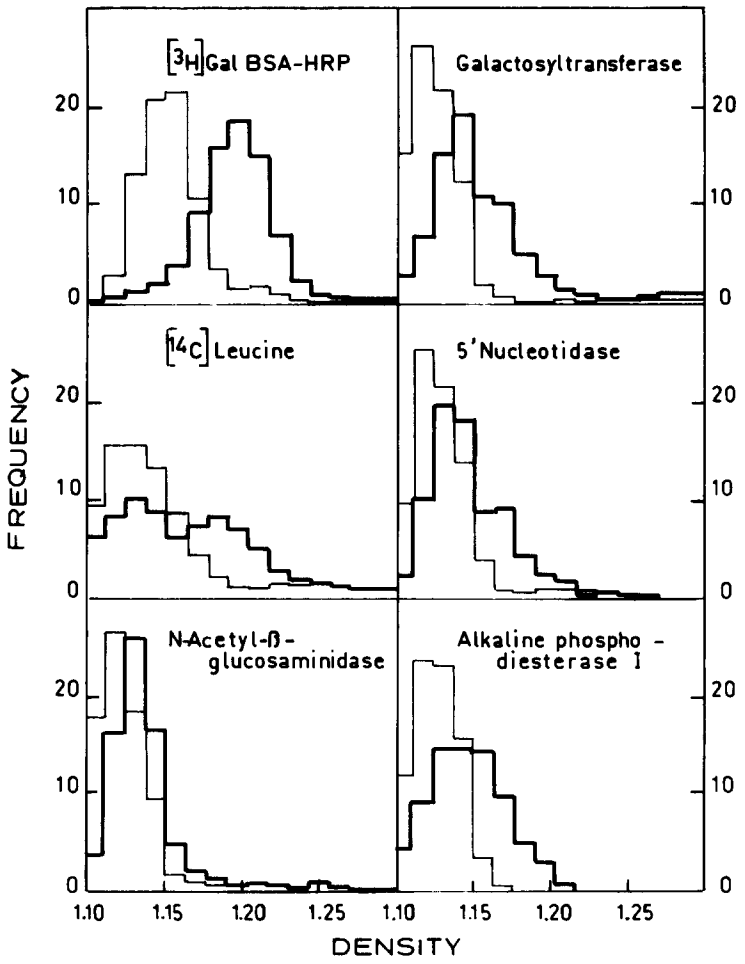

FIGURE 3 Density distributions after DAB-induced density shift of a L_1 pool. An L_1 pool instead of an LP_1 pool was incubated in the presence of DAB and H_2O_2 (thick lines) or of DAB alone (thin lines) in the same conditions as in Fig. 2. With respect to the L_1 pool, recoveries were between 72 and 106%. In this typical experiment, RSA (ratio of percentage of galBSA-HRP and that of [^{14}C]leucine) was in the L_1 pool and in the most enriched fraction after the DAB-induced density shift, 52.2 and 158.6, respectively.

major source of contamination in these preparations (see Discussion).

The experimental procedure for the isolation of galBSA-HRP-containing structures was next modified in an attempt to improve purification. First, the initial L fraction was washed more extensively; secondly, a tube slicer was systematically used to isolate the L_1 pool in view of lowering contamination during the collection procedure. To simplify the isolation procedure, the equilibration of the preparation incubated in the presence of DAB and H_2O_2 was performed on a discontinuous sucrose gradient and the material was collected at the 1.18–1.24 g/ml density interface.

As indicated in Table IV, the major difference from the results presented in Table III lies in an apparent 2.5-fold increase in the RSA of galBSA-HRP. This increase stems partly from an improvement of the yield and is partly due to the use of [^{35}S]methionine for assessing the protein content. As already observed for LP-derived material (see Table II), the use of [^{14}C]leucine as protein index resulted in a significant overestimate of the protein content. The ratio between the protein content based on [^{14}C]leucine incorporation and the colorimetric assay of Lowry et al. (27) was indeed 1.2 and 1.84 in the L fraction and L_1 pool, respectively. By contrast, the corresponding ratios using [^{35}S]methionine for protein determination were 1.00 and 1.04, respectively (Table IV).

Before assessing the enzyme composition of highly purified preparations of ligand-containing structures, the possibility of enzyme inactivation after incubation in DAB and H_2O_2 was investigated. Data of Table V show that some enzymes are indeed affected, the effect being largest for 5′-nucleotidase. The loss of activity calculated for the L_1 pool represents the maximal correction that could affect marker enzymes in the purified preparations, assuming that the inhibition occurs exclusively in the ligand-containing structures.

Two estimates of the enzymic composition of the ligand-containing structures have been made (Table VI). Both rest on the hypothesis that all enzymes are exclusively associated to ligand-containing structures, considering contamination by other organelles as negligible. The first estimate is not corrected for enzyme inactivation, while the second one introduces a correction assuming that the loss of activity in the L_1 preparation occurs exclusively in the ligand-containing structures.

Morphological Analysis of Purified Fractions

The preparations most enriched in galBSA-HRP containing structures were analyzed by electron microscopy (Fig. 4). For optimal identification of DAB-stained organelles, sections were first examined unstained. Of the total volume of structures recovered on the pellicle, DAB-stained organelles represented 83.4 ± 5.3% (mean ± SD), as estimated by morphometry. Contaminants were next analyzed in stained sections: they included rough and smooth vesicles, open membrane sheets, ferritin-loaded dense bodies (i.e., lysosomes), and mitochondria.

Stereological Analysis of galBSA-HRP–containing Structures on Intact Liver

The volume fraction and the membrane area of galBSA-HRP–containing structures in intact liver was measured by morphometry, 10 min after intravenous injection of 1 μg galBSA-HRP/g body wt, i.e., in similar conditions as for our fractionation experiments. This was done essentially to correlate the contribution of ligand-containing structures to cell protein with their contribution to the cell volume. In addition, the membrane area of the plasmalemma was also compared with that surrounding the ligand-containing structures.

As can be seen in Table VII, structures containing galBSA-HRP in these conditions occupied between 0.78 and 0.89% of the hepatocyte volume, depending on whether slightly stained structures were excluded or not from the estimate. Their membrane represented between 1,800 and 2,100 cm^2 per cm^3 of hepatocyte, a value corresponding to ~25% of the total surface of the hepatocyte pericellular membrane (6,700 cm^2/cm^3). The latter value is intermediate to 2,840 and 8,250 cm^2/cm^3 reported elsewhere for rat liver parenchyma (6,49). In our material, the sinusoidal domain with its microvillosities accounts for 57.3% of the total pericellular membrane.

TABLE III

Composition of Shifted Material from L_1 Pools

Marker	% of the homogenate	RSA
[^{14}C]leucine	0.070 ± 0.028	—
[^{3}H]galBSA-HRP	7.73 ± 2.68	118 ± 40
Galactosyltransferase	0.304 ± 0.247	3.86 ± 2.10
5′-Nucleotidase	0.069 ± 0.027	1.05 ± 0.40
Alkaline phosphodiesterase I	0.066 ± 0.063	0.73 ± 0.38
N-acetyl-β-glucosaminidase	0.037 ± 0.009	0.60 ± 0.29

L_1 pools from three different rats were incubated in DAB and H_2O_2 and equilibrated again in linear sucrose gradients. Values are means ± SD for the fraction with the highest galBSA-HRP specific activity in each experiment. These fractions were recovered between densities of 1.208 ± 0.005 and 1.221 ± 0.006 g/ml. [^{14}C]leucine incorporation was used to assess the protein content.

TABLE V

Effect of Cytochemistry and Equilibration in Sucrose Gradients on the Activity of Markers in the L_1 Pools

Marker	Content of L_1 pool	Recovery	Activity lost
	% *of homogenate*	% *of pool*	% *of homogenate*
[^{3}H]galBSA-HRP	13.38 ± 2.70	101.8 ± 25.0	—
Galactosyltransferase	2.24 ± 1.19	84.0 ± 23.0	0.342
5′-Nucleotidase	0.51 ± 0.21	56.9 ± 18.2	0.194
Alkaline phosphodiesterase I	0.76 ± 0.18	103.4 ± 35.4	−0.017
N-Acetyl-β-glucosaminidase	1.18 ± 0.65	78.1 ± 8.4	0.280

L_1 pools were incubated in DAB and H_2O_2 and equilibrated again in sucrose gradients. The recovery of marker activities in the gradient is used to evaluate the possible inactivation of enzymes after cytochemistry. The activity lost is the product of the yield in the L_1 pool, by 100 minus the recovery. Negative value indicates recovery above 100%. Values are means ± SD for three experiments.

DISCUSSION

Purification of Ligand-containing Structures

When applied to L_1 pools, the DAB-induced density shift procedure resulted in a 2.5- to 5.5-fold further purification of galBSA-HRP–containing structures, as compared with our previous results (35). When protein content was evaluated by the [^{35}S]methionine incorporation, which was in close agreement with determinations by the colorimetric procedure of Lowry et al. (27), an average RSA value of 267 was found for the purest preparations. Morphometry performed on one of these preparations showed that ~85% of the material could be identified as ligand-containing structures.

Our protocol was designed to optimize the purification of low density organelles involved in receptor-mediated endocytosis and may have selected a subpopulation of these structures. This possibility has to be seriously considered since these organelles are heterogenous in morphology (45, 47), topology (18), and physical properties (22, 30). Prior to delivery to lysosomes, galBSA is successively associated to two distinct types of organelles which are respectively recovered

TABLE IV

Subfractionation of L Fraction

	Protein			
	Lowry et al. (27)	[^{35}S]methionine	[^{3}H]galBSA-HRP	RSA
	%	%	%	
L fraction	4.02 ± 1.05	4.02 ± 0.45	23.0 ± 6.0	5.7 ± 2.1
L_1 pool	0.162 ± 0.075	0.168 ± 0.48	13.4 ± 3.3	82.1 ± 20.4
Shifted material	—	0.039 ± 0.005	10.4 ± 2.6	267 ± 48

The procedure reported in Table III for the isolation of shifted material was slightly modified. [^{35}S]methionine incorporation was selected as a protein index (see text). Same presentation as for Table II.

TABLE VI

Enzymic Composition of Ligand-containing Structures

Marker	Without correction for enzyme inactivation		With correction for enzyme inactivation	
	Yield	Contribution to the activity of the homogenate	Yield	Contribution to the activity of the homogenate
	% of homogenate	%	*% of homogenate*	%
[^{3}H]galBSA-HRP	10.444 ± 2.550	100.00	—	100.00
Galactosyltransferase	0.281 ± 0.008	2.69	0.623	5.97
5′-Nucleotidase	0.079 ± 0.009	0.76	0.273	2.61
Alkaline phosphodiesterase I	0.122 ± 0.052	1.17	0.105	1.01
N-Acetyl-β-glucosaminidase	0.098 ± 0.063	0.94	0.378	3.62

Calculations are based on the most enriched preparations (267 for the RSA in galBSA-HRP and 0.039% of protein as evaluated by methionine incorporation). The estimate of enzymic content is obtained by taking the yield in galBSA-HRP into account and assuming an exclusive association of markers to ligand-containing structures in the preparation. Evaluations are presented with and without correction for enzyme inactivation, based on Table V.

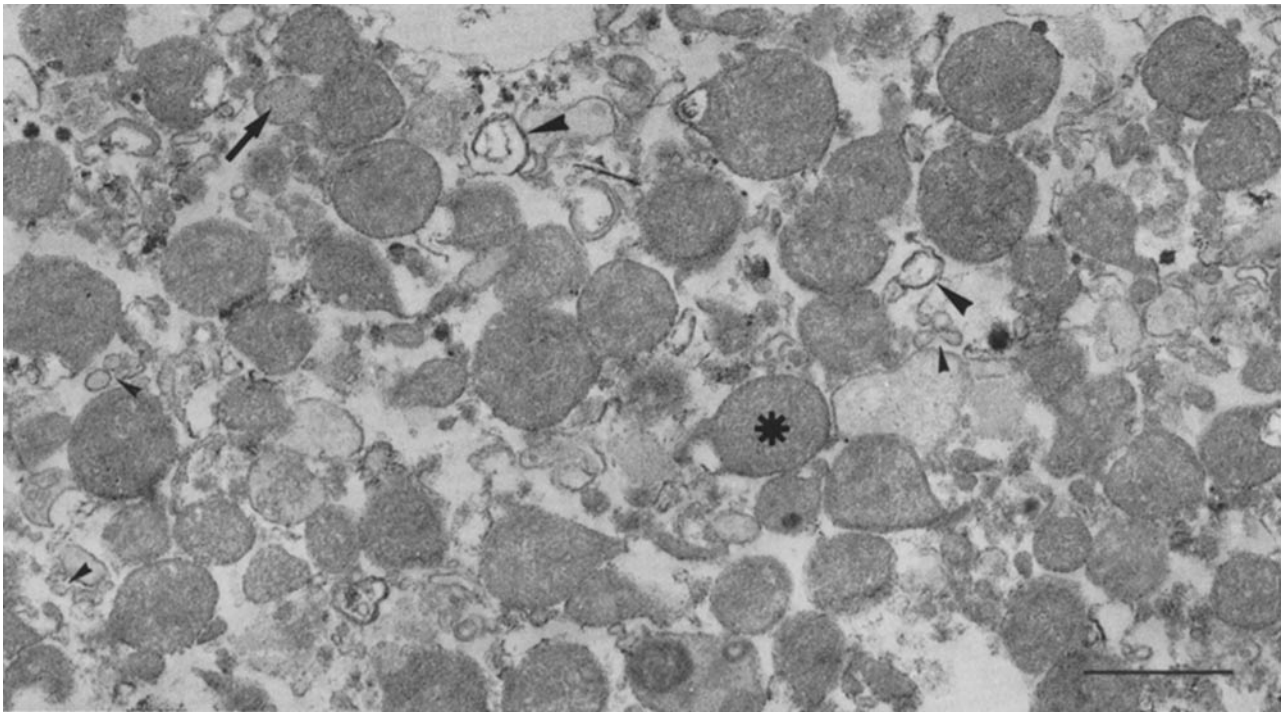

FIGURE 4 Purified preparation of ligand-containing structures. Unfixed L_1 pools were incubated in DAB and H_2O_2 and equilibrated again in sucrose gradients. Purified [^{3}H]galBSA-HRP–containing organelles were recovered at high density after the DAB-induced density shift. This preparation was fixed, filtered on Millipore^R, and examined at the electron microscope without further incubation in DAB and H_2O_2 or counterstaining with lead. Hence, electron-opaque profiles are nearly all ligand-containing organelles filled with DAB-osmium complexes (see asterisk for typical example). Contaminants include small clustered vesicles (small arrowheads), membrane fragments (large arrowheads), and ferritin-containing lysosomes (arrow). Average composition of similar preparations is described in Table VI. Bar, 0.5 μm. × 40,000.

in the P fraction, at densities ~1.13 g/ml in sucrose gradient, and in the L fraction, at densities ~1.11 g/ml (34, and J. Limet, personal communication). However, preliminary results in our laboratory indicate that the composition of these two populations are not substantially different (37).

Depending on whether or not slightly stained structures are included, the volume fraction of galBSA-HRP–positive structures evaluated in the liver (Table VII) would correspond to a RSA of only 110–130. Even if the nonhepatocytic compartment is taken into account, the corrected value would still be about half that found experimentally for the RSA of galBSA-HRP–containing structures. This is compatible with a low protein concentration in the newly formed endocytic structures, although other factors related to fixation and embedding, or differences in protein turnover, could be here at play.

Composition of Purified Preparations

Owing to the small amounts of enzymes present in the purified preparations of ligand-containing structures, it is clear that the concept of marker enzymes becomes particularly critical in our study. Two interpretations can be proposed: either the small amounts of marker enzymes result from a minor contamination of the preparation by structures other than those bearing galBSA-HRP, or these enzymes are truly associated to ligand-containing structures.

TABLE VII

GalBSA-HRP-containing Structures and Plasma Membrane: Stereological Data from Liver

	Membrane area per unit volume	Fractional volume
	cm^2/cm^3	%
HRP-positive structures		
Unequivocal labeling	1,800 ± 60	0.777 ± 0.103
Slight labeling	300 ± 80	0.112 ± 0.020
Total	2,100 ± 50	0.889 ± 0.099
Pericellular membrane		
Sinusoidal	3,840 ± 230	
Lateral	1,090 ± 40	
Biliary	1,770 ± 110	
Total	6,700 ± 190	

Animals were injected intravenously with 1 μg galBSA-HRP/g body wt 10 min before starting liver perfusion. Values are given ± SEM for the analyzed sample. The analyzed area was 2,700 μm^2 for the determination of HRP-positive structures and 900 μm^2 for measurements of pericellular membrane. For the latter, the lateral membrane is considered as extending from a tight junction at the biliary canaliculus to the first sectioned microvillosity at the blood front.

In the hypothesis of a minor contamination, the contribution to the protein content by each subcellular component can be analyzed according to Leighton et al. (25). Using the values previously reported (35) for the contribution of Golgi complex, plasma membrane, and lysosomes to the whole liver protein content, these structures would represent together <20% of the protein of preparations with an RSA of 267. This estimation was made without correction for enzyme inactivation since contaminants recovered at high densities should not contain DAB. Accordingly, the RSA of a pure fraction would be ~320, and these structures would represent ~0.3% of the liver protein, within the limits of our previous estimates (35). Owing to the limitation in the sensitivity of the assays, markers for mitochondria and endoplasmic reticulum, two major cell components, could not be followed. Electron microscopic examination showed that the main difference between our purest preparations derived from L_1 pools and those obtained from LP_1 pools (see Fig. 7 of reference 8) lies in a lower contamination by endoplasmic reticulum.

The alternative hypothesis implies the true association to ligand-containing structures of small quantities of classical markers for Golgi complex, plasma membrane, and lysosomes (summarized in Table VI), in the same way as small amounts of marker enzymes for the endoplasmic reticulum may be associated to Golgi stacks (20). Assuming no contamination by plasma membrane, lysosomes, and Golgi, at most 3% of plasma membrane markers, 3.5% of lysosomal markers, and 6% of Golgi markers could be associated to the structures that contain galBSA-HRP in our experimental protocol. In this hypothesis, the density shift of the marker enzymes (Fig. 3) would be concomitant with that of galBSA-HRP, as has been observed for "passive" constituents (8). The available data do not allow us to exclude any of the two hypotheses mentioned above, which are moreover not mutually exclusive.

Physiological Implications

The RSA of 5′-nucleotidase and alkaline phosphodiesterase I obtained in purified preparations of ligand-containing structures is much lower than in purified preparations of plasma membranes (19, 50), showing that only small amounts of plasma membrane markers may be present in the membrane of the ligand-containing structures, as compared with the pericellular membrane. Similarly, Merion and Sly (30) have recently reported that ligand-containing structures from human fibroblasts are largely dissociated from lactoperoxidase-iodinated cell surface proteins. Moreover, whereas at most 3% of plasma membrane markers can be attributed to structures that contain ligands in our experimental protocol, the membrane area of these structures corresponds to roughly a third of that of the pericellular membrane. For 5′-nucleotidase, the enzyme has been localized by cytochemistry along the entire pericellular membrane (12), where ~50% of the total cell activity is present (42). These results are not compatible with an identical marker enzyme composition of the plasmalemma and the membrane of ligand-containing structures. This conclusion is not necessarily in conflict with the numerous reports suggesting that the plasma membrane and the membrane of phagocytic or pinocytic origin are similar in polypeptide composition and marker enzyme activities (17, 29, 44, 51), since it is not established that pinocytosis, phagocytosis, and receptor-mediated endocytosis involve the same organelles. Recently, Dickson et al. (11) have also reported that the polypeptide composition of receptosomes is different from that of plasma membrane and lysosomes.

As the membrane of ligand-containing structures is presumably derived from the pericellular membrane, the lower content of membrane marker enzyme per unit surface implies some sorting mechanism. Membrane components could be prevented from entering the ligand-containing structures ("a priori" sorting) or could alternatively be rapidly and efficiently returned to the cell surface after entry ("a posteriori" sorting). An a priori sorting would imply that the specialized plasma membrane domains involved in receptor-mediated endocytosis are largely devoid of plasma membrane markers. Coated pits occupy only ~2% of the whole plasma membrane (2, 32), and selective exclusion of some plasma membrane components from coated pits has been documented (32). By double immunofluorescence localization, Stanley et al. (43) have recently reported that the localization of 5′-nucleotidase at the cell surface does not correspond to that of clathrin-coated pits.

Alternatively, the lower content of plasma membrane marker enzymes in the galBSA-HRP-containing structures, observed 10 min after ligand injection, could be explained by an a posteriori sorting. In this hypothesis, plasma membrane components, internalized with ligand-receptor complexes, would have already been returned to the cell surface, as is the asialoglycoprotein receptor (15, 38). Recycling of 5′-nucleotidase could occur through the tiny vesicles recently isolated by Luzio and Stanley (28). Preliminary results (37) indicate that 5 min after injection, the content in plasma membrane markers of galBSA-HRP-containing structures sedimenting in the LP fraction is already reduced.

The possible association of trace amounts of *N*-acetyl-β-glucosaminidase with ligand-containing structures can be easily explained. In our experiments, the ligand uptake was limited to 10 min in order to avoid significant transfer into lysosomes (35); nevertheless, we cannot exclude that some ligand-containing structures have already fused with lysosomes. In addition, lysosomal hydrolase activity could partially arise by receptor-mediated endocytosis of serum components (39).

The Golgi marker enzyme, galactosyltransferase, is present

in our purified preparations in larger proportion than marker enzymes for other structures. As to the origin and significance of galactosyltransferase in ligand-containing structures, this enzyme can be secreted (46) and endocytosed as discussed above for lysosomal hydrolases. Alternatively, ligands (5) including galactose-exposing proteins (8, 45, 47) have been found associated to lipoprotein-containing vesicles that could directly derive from the Golgi apparatus.

In conclusion, our data demonstrate that, after receptor-mediated internalization, galactose-exposing proteins are transferred into a unique subcellular compartment that appears structurally distinct from plasma membrane, lysosomes, and Golgi complex.

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