Shift of Equilibrium Density Induced by 3,3′-Diaminobenzidine Cytochemistry: A New Procedure for the Analysis and Purification of Peroxidase-containing Organelles

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ABSTRACT Galactosylated BSA (galBSA) and its conjugate to horseradish peroxidase (galBSA-HRP) enter the galactose-specific pathway of hepatocytes. 10 min after intravenous injection, structures containing either ligand sediment mostly between 33,000 and 3×10^6 $g \cdot$min (LP fraction) and have an equilibrium density of 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).

Such low density fractions, prepared from rats given galBSA-HRP, were incubated for 30 min at 25°C in 5.5 mM 3,3′-diaminobenzidine (DAB) and 11 mM H_2O_2 in buffered sucrose. Upon equilibration in a second sucrose gradient, the galBSA-HRP distribution shifted towards higher (~1.19 g/ml) density, but the bulk of protein remained at low density. In the absence of H_2O_2, galBSA-HRP distribution was also found at low density. As observed by electron microscopy, particles equilibrating at higher density after DAB cytochemistry were largely made of vesicles or tubules filled with DAB reaction product. The density shift of galBSA-HRP–containing organelles after incubation with DAB and H_2O_2 is attributed to the trapping of HRP-oxidized DAB inside the host organelles.

If the low density fractions isolated from a rat injected with [^{3}H]galBSA-HRP were mixed in vitro with similar fractions from another rat given [^{14}C]galBSA, the ^{3}H distribution shifted after DAB cytochemistry, but the ^{14}C distribution was essentially unaffected. By contrast, if both derivatives were injected simultaneously, a concomitant density shift was observed.

In conclusion, the DAB-induced density shift was specific to ligand-HRP–containing organelles. The potentials of the method include the purification of HRP-containing particles and the study of their association to ligands, fluid-phase tracers, or marker enzymes.

After receptor-mediated internalization (3, 28), ligands are rapidly transferred to an "intermediate compartment" (34, 37), often referred to as "endosomes" (14) or "receptosomes" (37). To assess the purity of subcellular fractions enriched in ligand-containing organelles, we recently used ligands conjugated to horseradish peroxidase (HRP),[1] an approach that proved successful in intact liver (29, 33). Glutaraldehyde-fixed fractions were incubated in 3,3′-diaminobenzidine (DAB) and H_2O_2 (12) and processed for electron microscopy. The organelles containing ligand-HRP were identified by the DAB reaction product (23). We report here that the DAB procedure can also be applied to unfixed subcellular fractions. Moreover, the accumulation of polymerized DAB inside HRP-containing organelles induces a major increase in their equilibrium density, while other organelles of the same fraction are essentially unaffected.

It has already been reported that cytochemical procedures may be applied to the isolation of specific organelles with endogenous marker enzymes, such as glucose 6-phosphatase (19) and 5′-nucleotidase (25) using lead cytochemistry, or

[1] *Abbreviations used in this paper:* DAB, 3,3′-diaminobenzidine; galBSA, galactosylated BSA; galBSA-HRP, galBSA conjugated to horseradish peroxidase (HRP).

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succinate dehydrogenase using tetrazolium cytochemistry (8). In comparison, the DAB-induced density shift takes advantage of a highly sensitive, exogenous enzyme, that can be introduced into specific organelles along the endocytic pathway by fluid-phase or receptor-mediated endocytosis (7, 23, 27, 29, 33). In addition, this procedure is simple, fairly reproducible, and proved useful to analyze and to purify ligand-HRP–containing organelles (24). This work has already been presented in abstract form (5).

MATERIALS AND METHODS

Preparation of Ligand and Ligand-HRP Conjugates: Galactosylated BSA (galBSA) was prepared according to Wilson (38), using a 96-h incubation in a 0.1 M borate buffer at pH 9. It was radiolabeled by reductive methylation (21), using [^{14}C]formaldehyde (The Radiochemical Centre, Amersham Corp., UK). Unlabeled galBSA was conjugated to HRP (type VI, Sigma Chemical Co., St. Louis, MO) after Nakane and Kawaoi (22) and labeled with sodium [^{3}H]borohydride (Amersham Corp.). The conjugate (galBSA-HRP) was cleared of unconjugated HRP and gal BSA, as well as of large polymers or aggregates, by isokinetic centrifugation in a sucrose gradient (17).

Characterization of Ligand-HRP Conjugates: The HRP/galBSA molar ratio, determined from the absorbance at 280 and 403 nm, was 1.4 and 1.7 for the two preparations used in this study. Ligand concentration was measured after Lowry et al. (20), using BSA as standard. The HRP activity of the conjugate was assayed using *N,N*-dimethyl-*p*-phenylenediamine (Merck, Darmstadt, Federal Republic of Germany) according to Straus (30) or DAB in a gelatin medium, as described by Herzog and Fahimi (16). This activity corresponded to 240–400 μg HRP (type VI)/mg galBSA. Radioactivity was measured in a Beckman LS 7500 DPM liquid scintillation counter (Beckman Instruments, Palo Alto, CA), after dissolution of the sample in 10 ml Aqualuma cocktail (Lumac Systems, Basel, Switzerland).

GalBSA-HRP conjugate, like galBSA, was recognized almost exclusively by the galactose-specific receptor of the hepatocytes since: (*a*) after intravenous injection to rats, the conjugate was rapidly cleared from the blood by the liver, (*b*) after cytochemistry, galBSA-HRP was found almost exclusively in the hepatocytes by light and electron microscopy, and (*c*) the uptake of galBSA-HRP by cultured rat hepatocytes could be inhibited by a large excess of asialofetuin or galBSA, but not by native BSA. In addition, the subcellular distribution of both ligands after differential and isopycnic centrifugations was comparable, indicating similar processing by the cell (23).

Preparation of DAB Solutions: 3,3′-Diaminobenzidine tetrahydrochloride (type II, lot 88C-03201, Sigma Chemical Co.) was dissolved at a concentration of 2.56 mg/ml (7.1 mM) in 0.74 M sucrose (density: 1.1 g/ml), buffered with 3 mM imidazole/HCl (31) and the pH was carefully adjusted to 7.0 with concentrated NaOH. This brownish solution was cleared by filtration on two layers of a 0.2-μm pore Metricel^{R} filter (Gelman, Ann Arbor, MI) and used extemporaneously. The absorbance at 280 nm of the filtrate diluted 50-fold was 1.63 ± 0.06. The stability of DAB solutions in the presence of H_2O_2 was routinely checked before use. Solutions that rapidly turned brown were discarded.

Subcellular Fractionation: Rats (200–250 g) were injected intravenously with 1 μg/g body wt [^{3}H]galBSA-HRP (126 nCi/μg) or [^{14}C]galBSA (7 nCi/μg). 10 min after injection, the liver was perfused by the portal vein with H16 tissue culture medium (Gibco Biocult, Paisley, UK). The liver homogenate was fractionated by differential centrifugation (9) to successively pellet N (10,000 *g*·min), M (33,000 *g*·min), and combined LP (3 × 10^6 *g*·min) fractions.[2] LP fractions (5ml) were layered on the top of a 32-ml linear sucrose gradient (from 1.1 to 1.3 g/ml in density), buffered with 3 mM imidazole/HCl, pH 7.0. After centrifugation for 24 × 10^6 *g*·min in a VTi 50 rotor (Beckman Instruments), 14 fractions were collected and analyzed as previously described (23). The two fractions containing the highest radioactivity (average density: 1.12 g/ml) were pooled and will be further referred to as LP_1 pool.

For the diaminobenzidine procedure, 1.75 ml of the LP_1 pool was added to 6.25 ml of the DAB solution (final concentration: 5.5 mM, nominal). The reaction was started by the addition of 50 μl of 6% H_2O_2, prepared extemporaneously by dilution of a 30% H_2O_2 stock solution in sucrose-imidazole, and performed for 30 min at 25°C, in the dark, under gentle agitation. The H_2O_2 concentration in the incubation medium (11 mM) was measured by the $TiOSO_4$ colorimetric assay (4). The stock H_2O_2 concentration was verified by $KMnO_4$ titration (32).

After incubation, 5 ml of the suspension was equilibrated in a second linear sucrose gradient (density, 1.13–1.30 g/ml). Fractions therefrom were analyzed for density, radioactivity, and enzyme activities. Owing to an interference of DAB with the protein assay of Lowry et al. (20), liver protein was labeled in some experiments by a pulse of [^{14}C]leucine (125 μCi) injected intraperitoneally 40 h before sacrifice. In such conditions, values of 3.6 ± 0.7 nCi/mg liver protein and 431 ± 3 nCi/ml plasma were obtained (*n* = 3).

Electron Microscopy: The subcellular fractions were fixed by the slow addition of glutaraldehyde (7.5% in 0.1 M cacodylate, pH 7.4) to a final concentration of 1.5% (18) and kept overnight at 4°C. About 100 μg of protein of each fraction of interest was then applied over a 1-cm^2 Millipore^{R} filter, 50-nm nominal pore size (Millipore Corp., Bedford, MA), and filtered under one to two bars (2). The retention of the material on the filter was ascertained by measuring the radioactivity of the filtrate and was >90%. The pellicle was postfixed for 1 h at 4°C in a solution containing 1% osmium tetroxide and 1% potassium ferrocyanide and dehydraded in graded alcohol. After dissolution of the nitrocellulose filter in propylene oxide, the pellicle was embedded in the mixture described by Spurr (26). Ultrathin sections were examined unstained under 60 kV in a EM 301 Philips electron microscope.

RESULTS

Effect of DAB Cytochemistry on the Density Distribution of galBSA-HRP

The density distributions of [^{3}H]galBSA-HRP- or [^{14}C]-galBSA-containing organelles, sedimenting in a LP fraction, are illustrated at Fig. 1. 10 min after intravenous injection, both ligands are associated to structures that equilibrate in the low density region of the sucrose gradient and that are dissociated from the bulk of protein (23). For DAB cytochemistry, the LP_1 pool corresponding to the peak of the [^{3}H]-galBSA-HRP distribution (density, 1.11–1.13 g/ml) was incubated at 25°C in buffered sucrose (density, 1.1 g/ml), containing 5.5 mM DAB and 11 mM H_2O_2. This resulted in the development of a brownish color. In the absence of H_2O_2, the mixture remained yellowish. After 30 min incubation, the granules were equilibrated again in a sucrose gradient.

If H_2O_2 was included in the incubation medium, the density distribution of [^{3}H]galBSA-HRP shifted towards a heavier density, upon equilibration in a second sucrose gradient (Fig. 2). The increment in median equilibrium density was 0.054 g/ml (SD: 0.004, *n* = 4). By contrast, the bulk of [^{14}C]leucine-

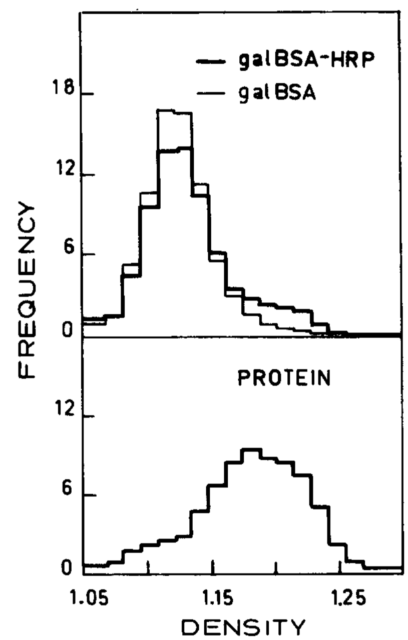

FIGURE 1 Density distributions of [^{3}H]galBSA-HRP or [^{14}C]galBSA. [^{3}H]galBSA-HRP or [^{14}C]galBSA were injected intravenously (1 μg/g body wt of either ligand) into rats, 10 min before sacrifice. LP fractions were prepared and layered on top of a sucrose gradient. Protein was determined by the procedure of Lowry et al. (20). Distributions are averages of seven experiments. Recoveries were 108.4, 119.7, and 98.5% of the LP fraction for [^{3}H]galBSA-HRP, [^{14}C]galBSA, and protein, respectively.

[2] See Materials and Methods of the companion paper (24) for an explanation of the fraction nomenclature.

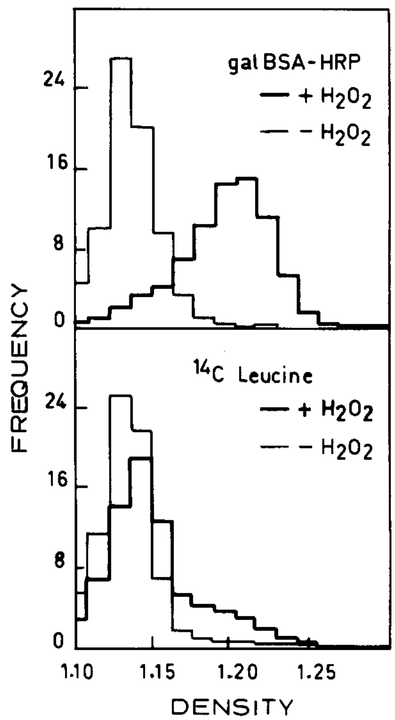

FIGURE 2 Density distribution of [^{3}H]galBSA-HRP after DAB cytochemistry. Rats were given a pulse of [^{14}C]leucine 40 h before sacrifice, so as to label liver protein. [^{3}H]galBSA-HRP was injected intravenously (1 μg/g body wt), and the liver was homogenized 10 min thereafter. An LP fraction was layered over a sucrose gradient and centrifuged. The fractions equilibrating between 1.11 and 1.13 g/ml in density were pooled (LP_1 pool), incubated for 30 min in 5.5 mM DAB in the presence or absence of 11 mM H_2O_2, and equilibrated again in a sucrose gradient (mean values of two experiments). Average recoveries with respect to the LP_1 pool were 95.8% for [^{3}H]galBSA-HRP and 105.0% for [^{14}C]leucine.

labeled protein remained in the low density region, although a minor component shifted towards a heavier density, probably representing protein associated with ligand-containing structures. If H_2O_2 was omitted, the distributions of [^{3}H]-galBSA-HRP- and [^{14}C]leucine-labeled protein remained at low density. In this case, distributions were only slightly shifted (~0.01 g/ml in density) in comparison to fractions incubated without DAB.

Origin and Factors of the DAB-induced Density Shift

If galBSA-HRP was replaced by galBSA, or if either DAB or H_2O_2 was omitted from the incubation medium, no significant change in the color or the distribution of the ligand was observed. Therefore, the major shift of the equilibrium density of ligand-HRP conjugates after incubation in DAB and H_2O_2 stems from the oxidation of DAB by HRP. These experiments also indicate that no significant endogenous H_2O_2 or peroxidatic activity was present in the low density fractions.

The relation between the association of oxidized-polymerized DAB to the granules and the density shift was further studied. First, the density distribution of oxidized DAB after cytochemistry was compared with that of galBSA-HRP. For this purpose, LP_1 pools were prepared from rats injected with either galBSA-HRP or galBSA. After incubation in presence of DAB and H_2O_2, the preparations were equilibrated in a second sucrose gradient and the absorbance at 465 nm was measured. The difference in the absorbance of corresponding fractions was taken as an estimate of HRP-oxidized DAB. As can be seen in Fig. 3, the distributions of oxidized DAB and galBSA-HRP were very similar. Next, DAB was oxidized and polymerized with ferricyanide (13) or by soluble HRP and H_2O_2 and then layered on the top of a sucrose gradient. After centrifugation, all brown material was found as a pellet below the heavy sucrose cushion (density, 1.34 g/ml). This demonstrates that the equilibrium density of oxidized and polymerized DAB is >1.34 g/ml and that accumulation of this compound inside vesicles should result in a substantial increase of their equilibrium density.

Changing parameters of the DAB reaction was shown to influence the extent of the density shift. Reduction of the amount of galBSA-HRP injected, of incubation time, or of DAB concentration all resulted in a smaller density shift of the galBSA-HRP distribution (Fig. 4). In our standard conditions (5.5 mM DAB), the DAB concentration was not largely decreased at the end of the incubation, as measured by performing the gelatin assay (16) in the presence of a constant HRP concentration. In the same conditions, H_2O_2 concentration rapidly dropped, with a half-life of ~10 min. However, repeated addition of H_2O_2 during the incubation did not enhance the density shift. We also tested if the one-step addition of H_2O_2 could be replaced to advantage by a continuous production of H_2O_2, using a glucose/glucose oxidase system. A similar DAB-induced density shift could be produced but the procedure was less reproducible.

Finally, to assess the accessibility of DAB into membrane-closed organelles, LP_1 pools from rats injected with galBSA-HRP were assayed for HRP activity, in a medium containing 0.74 M sucrose, 3 mM imidazole/HCl, pH 7.0, 5.5 mM DAB, 11 mM H_2O_2, and 0.1% gelatin, in the presence or absence of 0.1% Triton X-100. While Triton X-100 had no significant effect on the activity of free HRP, its addition to the organelle suspension caused a 10-fold increase of the HRP activity. In our opinion, these observations most likely reflect a restriction in the diffusion of soluble DAB through intact biological membranes, although an inhibition of HRP by the reaction product trapped inside the particles could also explain our results.

Specificity of the DAB-induced Density Shift

To assess the specificity of the density shift, in vitro mixing experiments were performed. A rat was injected with [^{3}H]-galBSA-HRP and another one received [^{14}C]galBSA. LP_1

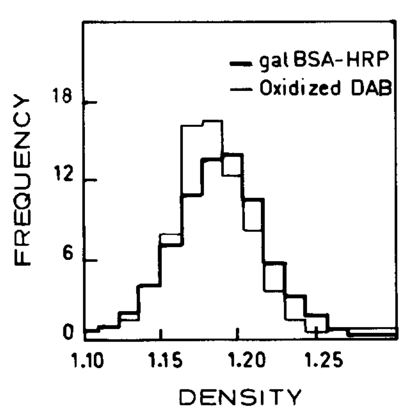

FIGURE 3 Distribution of oxidized DAB after density equilibration. LP_1 pools were prepared from rats injected with either [^{3}H]-galBSA-HRP or [^{14}C]-galBSA. After incubation in DAB and H_2O_2, they were equilibrated again in a sucrose gradient. The distribution of HRP-oxidized DAB was estimated from the absorbance at 465 nm (see text).

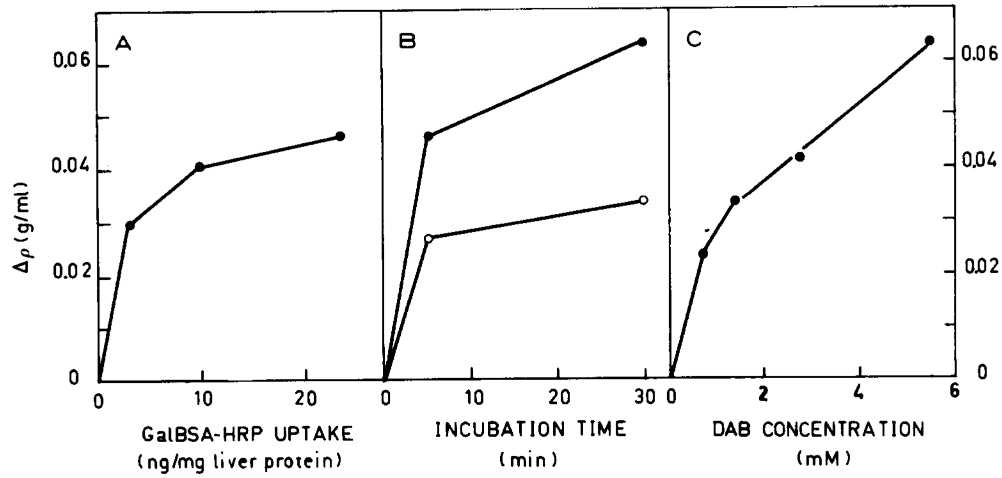

FIGURE 4 Kinetics and parameters of the DAB-induced density shift. Same preparations as in Fig. 2. The ordinate is the difference in the median equilibrium density of [^{3}H]galBSA-HRP after incubation in DAB with and without H_2O_2. (*A*) Effect of the amount of galBSA-HRP. The plateau reached at 10 ng/mg of liver protein corresponds to the injection of 250 ng/g body wt. (*B*) Effect of incubation time. DAB concentrations used are 1.4 mM (open circles), and 5.5 mM (closed circles). (*C*) Effect of DAB concentration.

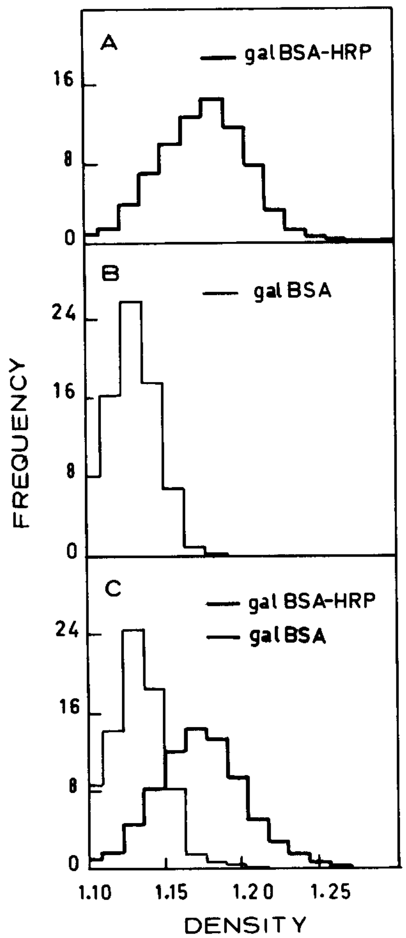

FIGURE 5 In vitro mixing experiment. LP_1 pools were prepared from rats given either [^{3}H]galBSA-HRP or [^{14}C]galBSA 10 min before sacrifice and incubated in DAB and H_2O_2, separately (*A* and *B*) or together (*C*). Average recoveries with respect to the LP_1 pool were 97.5% for [^{3}H]-galBSA-HRP and 97.0% for [^{14}C]galBSA.

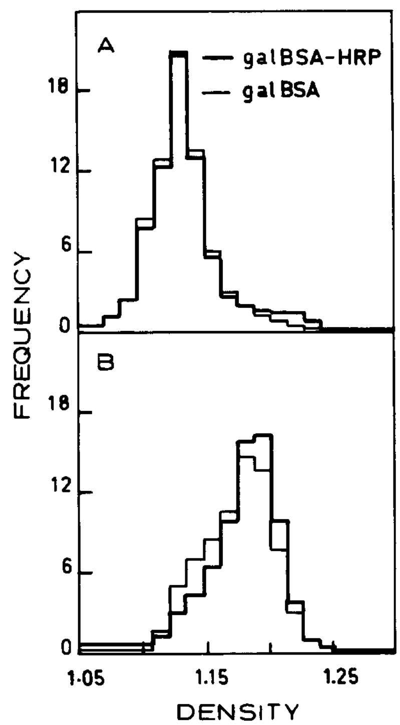

FIGURE 6 In vivo mixing experiment. A rat received a simultaneous injection of [^{3}H]-galBSA-HRP and [^{14}C]-galBSA (0.5 μg/g body wt of each derivative), 10 min before sacrifice. (*A*) An LP fraction was equilibrated in a sucrose gradient; (*B*) the LP_1 pool isolated from *A* was incubated in the presence of DAB and H_2O_2 for 30 min, and equilibrated again in a sucrose gradient. Average recoveries were 101.5% for [^{3}H]galBSA-HRP and 103.5% for [^{14}C]galBSA, with respect to the sample layered on the gradient.

pools were isolated as previously described. First, they were incubated separately in DAB and H_2O_2 and equilibrated again in a sucrose gradient. As in the previous experiments, the distribution of galBSA-HRP was shifted to a higher density and that of galBSA was not appreciably affected. Next, the LP_1 pools from the two animals were mixed in vitro, incubated together in DAB and H_2O_2, and equilibrated again in a sucrose gradient. The distribution of [^{3}H]galBSA-HRP was shifted and that of [^{14}C]galBSA remained essentially unchanged (Fig. 5). This demonstrates that the DAB-induced density shift is specific to HRP-containing organelles and that no appreciable agglutination, or fusion between similar organelles, occurred in our experimental conditions. Therefore, the DAB-induced density shift can be used to demonstrate the dissociation of two similar and yet distinct subcellular populations.

By contrast, if free HRP (1 μg/ml) was added to the LP_1 pool from a rat injected with galBSA and incubated with DAB and H_2O_2, DAB was immediately oxidized and the radioactivity was recovered with DAB as a pellet, below a density of 1.34 g/ml. This indicates that free HRP caused the agglutination of particles and that the specificity of the DAB-induced density shift may be jeopardized in the presence of soluble HRP and possibly of other heme proteins.

Concomitant Density Shift of Two Ligands

A rat received a simultaneous injection of [^{3}H]galBSA-HRP and [^{14}C]galBSA. An LP fraction was first equilibrated in a sucrose gradient. The density distribution of both ligands was similar and displayed a peak in the low density region. The LP_1 pool was incubated in DAB and H_2O_2 and equilibrated again in a sucrose gradient. The density distributions of galBSA-HRP and galBSA shifted concomitantly (Fig. 6).

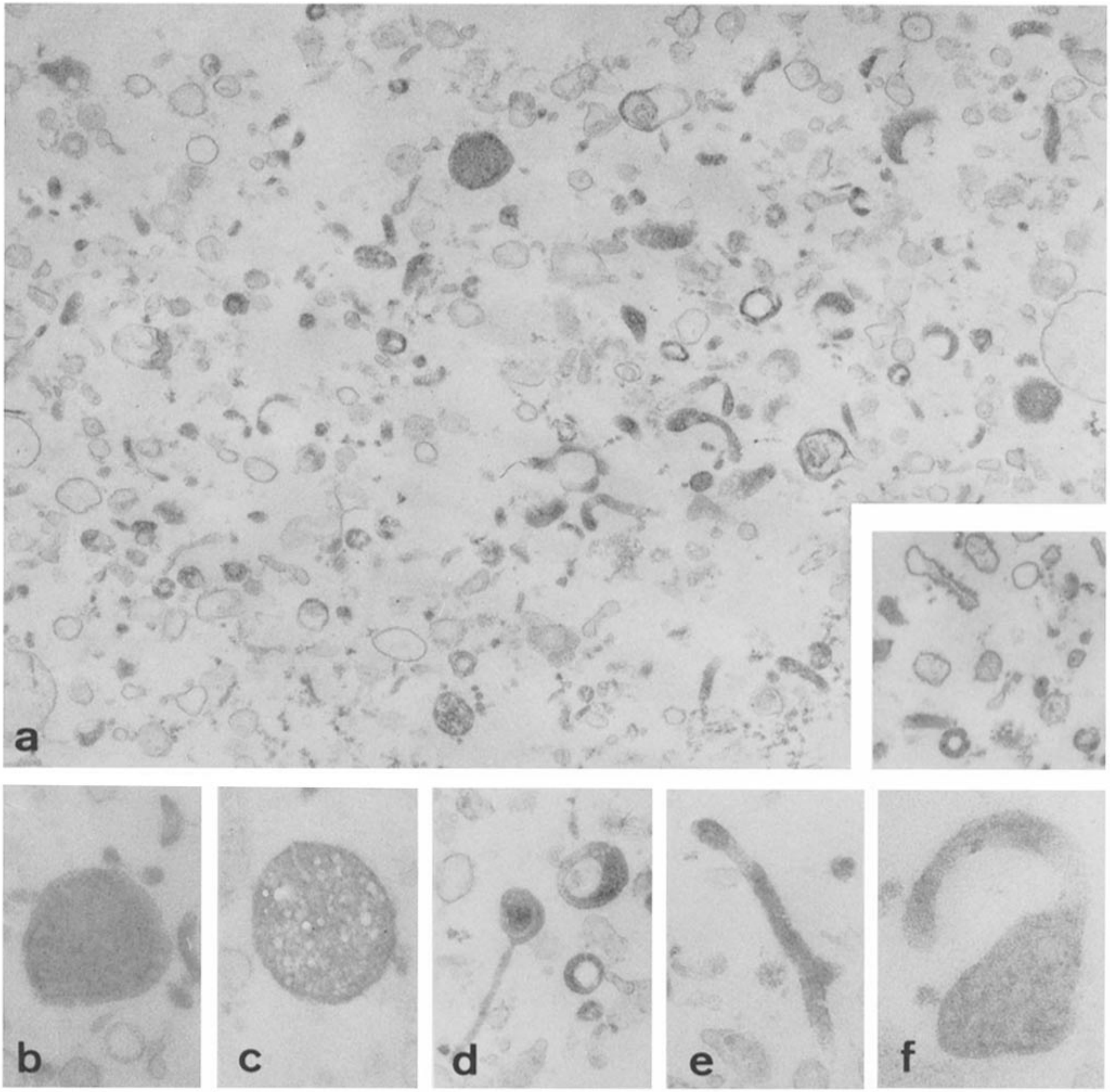

FIGURE 7 Ultrastructural demonstration of HRP-containing organelles after DAB-induced density shift. Unfixed LP_1 pools were incubated in DAB and H_2O_2 and equilibrated again in sucrose gradients, as described in Fig. 2. Fractions enriched in [^{3}H]galBSA-HRP–containing organelles were recovered at high density after the DAB-induced density shift. The 1.23-g/ml fraction was fixed, filtered on Millipore^R, and processed for electron microscopy without further incubation in DAB and H_2O_2. Ultrathin sections, perpendicular to the filter surface, were usually examined unstained. The field shown in *a* is a general view of the pellicle (filter is at bottom). Notice the abundance and heterogeneity of the organelles filled with DAB reaction product. Enlargements (*b–f*) illustrate various aspects of isolated ligand-containing structures. They include sections through small and large vesicles (*b*), often containing numerous small (10–20 nm) electron-lucent spheres (*c*), cupshaped vesicles (*d*), and elongated or tortuous tubules (*e*) frequently connected to small (*d*) and large profiles (*f*). Contaminants, which are identified in stained sections (*inset* to *a*), are largely made of endoplasmic reticulum elements. (*a*) × 44,000; *inset*: × 44,000, (*b*, *c*, and *f*) × 75,000; (*d*) × 58,000; (*e*) × 71,000.

Other structures remained essentially at the initial low density, as indicated by the distributions of marker enzymes for the plasma membrane, lysosomes, and the Golgi complex (not shown). The density distributions of these marker enzymes are presented in detail in the companion paper (24).

Since the density shift induced by galBSA-HRP ("active" ligand) caused a concomitant density shift of galBSA ("passive" ligand), but not of other components, this experiment demonstrated that both derivatives were localized within the same organelles. This result illustrates that the DAB shift procedure could be used to test directly whether components with overlapping distributions in a fractionation system are truly associated to the same host particles, provided agglutination of granules can be excluded (see Discussion).

Electron Microscopy

After the DAB-induced density shift, the material recovered at the density of 1.23 g/ml was fixed, processed for electron

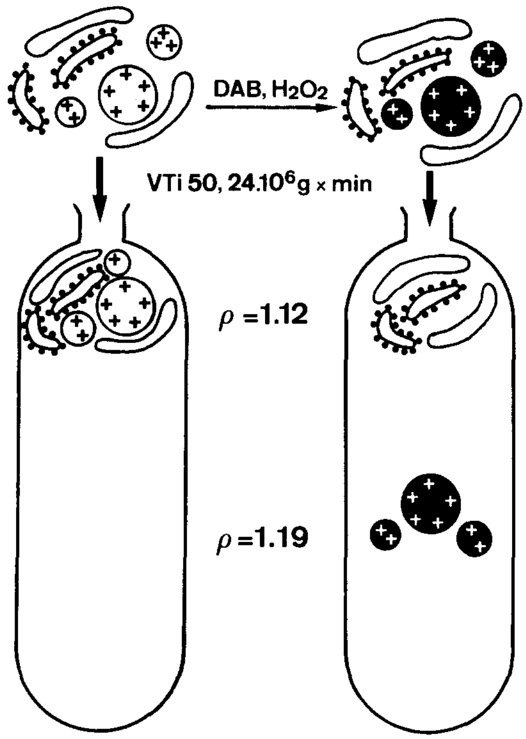

FIGURE 8 Model for the DAB-induced density shift. In the absence of HRP or H_2O_2, DAB diffuses slowly in and out all organelles. In those vesicles that contain HRP, DAB is oxidized and polymerized in the presence of H_2O_2 and remains trapped. Since the equilibrium density of polymerized DAB is high (>1.34 g/ml), this results in a large, specific increase in the equilibrium density of HRP-containing vesicles.

microscopy without further incubation in DAB and H_2O_2, and examined unstained (Fig. 7). For comparison, LP_1 pools were first fixed and then incubated in DAB and H_2O_2. In both preparations, ligand-containing structures were identified as vesicles and tubules filled with electron dense material. However, application of the cytochemical procedure of Graham and Karnovsky (12) to unfixed organelles resulted in a lower contrast than with fixed specimens (see Fig. 7 of reference 23 for comparison). This is attributed to the restricted diffusion of soluble DAB through intact membranes, as previously discussed. Whereas DAB-stained structures represented a small proportion of the organelles occurring in the LP_1 pool (not shown), they constituted a major component (45–53% of the total volume of particles) of the high density fractions, after the DAB-induced density shift.

DISCUSSION

Physical Basis of the Density Shift

Several lines of evidence indicate that the shift in the equilibrium density of HRP-containing organelles results from the trapping of DAB that has been oxidized and polymerized inside these organelles by HRP: (*a*) the density shift requires the simultaneous presence of HRP, DAB, and H_2O_2, and increases as a function of HRP and DAB concentrations; (*b*) the density distribution of ligand-HRP conjugate after cytochemistry was similar to that of HRP-oxidized DAB; (*c*) as observed by electron microscopy, a large proportion of the structures equilibrating at higher density after cytochemistry were filled with DAB reaction product; and (*d*) the equilibrium density of polymerized DAB is >1.34 g/ml.

Our interpretation of the physical basis of the DAB-induced density shift is outlined in Fig. 8. The model accounts for the major increase in density of HRP-containing organelles, and for the specificity of the density shift, which is restricted to HRP-containing structures. Although our results imply that DAB gains access inside unfixed closed structures, the latency of HRP towards DAB in preparations of intact granules points to a restriction of the diffusion of DAB through biological membranes. This is not surprising in view of the polar character of the DAB molecule, and explains most probably that the concentration (5.5 mM) required for obtaining a large density shift is 10-fold the optimal concentration of the assay for free HRP (16).

Validity of the Method

In our hands, the DAB-induced density shift appeared as a fairly simple, reproducible, and specific procedure. The specificity of the density shift to ligand-HRP–containing structures was demonstrated by in vitro mixing experiments, a criterion already used by Leskes et al. (19). In addition, only a minor portion of the protein shifted after cytochemistry, and the distribution of several marker enzymes present in the initial preparation was largely unaffected (24).

However, the specificity of the procedure can be jeopardized. The preparation should be virtually free of soluble, and possibly externally exposed or adsorbed, HRP activity. As mentioned in Results, free HRP rapidly oxidized DAB outside the organelles, leading to agglutination of the preparation. This is an important caveat, since it may preclude the use of the method in preparations containing soluble HRP or soluble HRP conjugates. In addition, the possibility of obtaining a specific density shift when HRP is associated to open structures, such as membrane fragments, is not yet documented. Furthermore, subcellular fractions of different purity, composition, or origin may exhibit specific agglutinating properties or contain various amounts of endogenous H_2O_2 or HRP activity. As a consequence, it should be stressed that while a dissociation in the distributions of different markers demonstrated by the DAB shift procedure may be considered as conclusive, the evidence of the association of two components based on the criterion of concomitant DAB-induced density shift requires that agglutination can be excluded, for example by in vitro mixing experiments.

Analytical and Preparative Applications to Subcellular Organelles

Most purification procedures based on specific density alterations induce a primary change in the density of specific organelles, resulting from the in vivo accumulation of light (11, 35, 36) or heavy material (15). In contrast, the density alteration described in this and other (8, 19, 25) papers is performed in vitro on subcellular organelles whose equilibrium density is initially unaffected.

Since DAB cytochemistry induces a shift towards heavier densities, this procedure is particularly suited for the purification of organelles equilibrating at a low density. Indeed, a two-step purification protocol can be followed in this case. A first density equilibration can be used to clear the preparation from organelles equilibrating in the heavy region of the gradient. After DAB cytochemistry is performed on the particles equilibrating at low density, a second isopycnic centrifugation will place HRP-containing particles at their new (heavier) equilibrium density, in a region of the gradient devoid of other particles. Particles without HRP will remain at their initial density. Application of this strategy to the purification of the low density ligand-containing structures is reported in the companion paper (24).

Examination of the results reported here shows that the shifted distributions of HRP-containing structures are more disperse than the initial ones. This may affect the yield of a purification. Assuming a Poisson distribution for the exogenous marker molecules throughout a population of vesicles, considerable variation may occur in the marker content of individual vesicles, if the average number of molecules per vesicle is small. For example, if an average of 10 molecules of HRP-conjugate have been introduced per low density vesicle, the standard deviation will be 3.2 molecules per vesicle. Such variation in the content of "active" ligand may account for the observed dispersion in the distribution of galBSA-HRP-containing vesicles, after DAB cytochemistry.

Our procedure can also be applied for analytical purposes, in the same way as density shifts induced by other compounds, such as digitonin or pyrophosphate (1). In the companion paper, we report the application of DAB-induced density shift to the analysis of highly purified ligand-containing structures, using galBSA-HRP (24). The same procedure was also applicable to the study of the lysosomal membrane of fibroblasts after HRP uptake by fluid-phase endocytosis and accumulation in lysosomes (10). Finally, we have also applied the procedure of the DAB-induced density shift to the study of ligand sorting in rat hepatocytes. Polymeric immunoglobulin A, a ligand that is transferred into bile and galBSA-HRP, which is digested in lysosomes, were injected simultaneously at various times before sacrifice. The concomitant shift of polymeric immunoglobulin A ("passive" ligand) and galBSA-HRP ("active" ligand) was used to assess their association to the same structure (6).

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