Catabolism of Hemoglobin–Haptoglobin Complex in Microsome Subfractions

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After internalization of hemoglobin–haptoglobin complex (Hb–Hp) *via* receptor-mediated endocytosis (RME) into liver parenchymal cells, organelles containing the complex distribute in the microsome fraction (Ms). Prior to the catabolism, Hb–Hp dissociates symmetrically into two 82000-dalton (82 kDa) subunits. In the present investigation, the first event of Hb–Hp metabolism in Ms were further examined after [^{3}H-heme, ^{14}C-glogin]Hb–Ho or [^{125}I-Hb]Hp injection to rats. Shortly after the internalization of Hb–Hp, this complex in Ms was intact. At 60 min after injection, radioactive materials of Ms extracted by freezing and thawing (F&T) with yield of 15% were composed of Hb–Hp, 82 kilodaltons (kDa) subunits and Hb metabolites with a ratio of 1:6:13. The heme metabolites were identified as [^{3}H]bilirubin by high performance liquid chromatography (HPLC). The ratio of Hb–Hp/82 kDa subunits/Hb metabolites in microsome residue of the F&T was 40:8:1. The radioactivity in Ms at 60 min localized microsome subfraction except Golgi light fraction. In electron microscope radioautography of microsome subfraction using [^{125}I]Hb–Hp, silver grains were observed over or within morphologically heterogenous vesicles, *e.g.* vesicles containing very low density lipoprotein (VLDL) particles with appendage like multi-vesicular body (MVB) or compartment of uncoupling of receptor and ligand (CURL) in Goligi light and intermediate fractions.

These studies suggest that Hb–Hp internalized by RME is dissociated symmetrically into two 82 kDa subunits in organelles of Ms, and that organelles with MVB or CURL-like structures are associated with Hb–Hp metabolism.

Keywords hemoglobin–haptoglobin complex; heme; bilirubin; microsome; Golgi; compartment of uncoupling of receptor and ligand (CURL); multi-vesicular body (MVB)

Hemoglobin released from erythrocytes into the circulation by intravascular hemolysis binds immediately with haptoglobin, a serum glycoprotein, and forms a stable hemoglobin–haptoglobin (Hb–Hp) complex. Our previous work demonstrated that the Hb–Hp thus formed is removed from the ciculation by a receptor specific for the molecule in the liver parenchymal cell membranes.[1] The Hb–Hp molecules are first concentrated in organelles recovered in microsome fraction (Ms) in a substantially intact form. The organelles containing Hb–Hp progressively acquired a higher density in Percoll and anodic mobility in free-flow electrophoresis, and the complex first dissociated symmetrically into two 82 kDa subunits with intact heme at an early stage of degradation.[2] In subsequent stages, the organelles containing only heme moiety were separated from mitochondria–lysosomal fraction (Mt–Ly) by free-flow electrophoresis.[3] In the present investigation, we carried out further study on the early stage of Hb–Hp metabolism in Ms and found that the dissociation site of the Hb–Hp into 82 kDa subunits and the morphology of organelles internalized the complex in Ms of rat liver cells.

These results suggest that Hb–Hp is converted to two 82 kDa subunits in organelles of Ms shortly after receptor-mediated endocytosis (RME) and that multi-vesicular body (MVB) or compartment of uncoupling of receptor and ligand (CURL)-like organelles are concerned with Hb–Hp metabolism.

Materials and Methods

Preparation of the Biological Materials [^{3}H-heme, ^{14}C-globin]Hb was synthesized by incubating the reticulocytes from newborn rats with 5.0 mCi of δ-amino[2,3-^{3}H]laevulinic acid (CEA, 318 mCi/mmol) and 0.1 mCi of L-[U−^{14}C]Leu (NEN, 300 mCi/mmol) for 8 h at 37 °C as described previously.[4] The specific ^{3}H and ^{14}C radioactivities of doubly labeled Hb were 206.85×10^4 dpm/mg protein and 22.27×10^4 dpm/mg protein, respectively. The ratio of ^{3}H to ^{14}C radioactivity was 9.3. It has been reported that fetal Hb was not detected in rat erythrocytes after 18 d of gestation or in newborn.[5] Hb concentration was determined by the method of Drabkin and Austin.[6] [^{3}H-heme, ^{14}C-globin]Hb–Hp was prepared by adding the doubly labeled Hb to Hp partially purified from rat serum according to the method of Hamaguchi.[7] Since a Hb molecule (M_r 68000) binds to a Hp molecule (M_r 100000) to form Hb–Hp, the mixture of Hb and Hp solution was prepared in the ratio of 1 to 1.47 mg. The ratio was determined because analyses of gel filtration and polyacrylamide gel electrophoresis showed that more than 95% of Hb in the injected preparation bound with Hp to form the stable complex. [^{125}I]Hb was prepared by the method of Sonoda and Schlamowitz,[8] and the specific radioactivity was 1.8×10^8 cpm/mg protein. [^{125}I]Hb–Hp was prepared by the above method.

Uptake of [^{3}H-heme, ^{14}C-globin]Hb–Hp into Liver Uptake of double-labeled Hb–Hp (0.1 or 0.5 mg as Hb/100 g of body weight) to rat liver was carried out by our previous method.[3]

Subfractionation of Microsomes Golgi and endoplasmic reticulum (ER) fractions were isolated according to Ehrenreich *et al.*[9] with slight modification. Rats were given ethanol (50%, w/w) by stomach tube 90 min prior to killing, and at various times the animals were given an injection of [^{3}H-heme, ^{14}C-globin]Hb–Hp (0.1 or 0.5 mg as Hb/100 g of body weight) *via* the portal vein. Animals were treated by perfusion with 2.5 mM triethanolamine-acetate (TEA) buffer, pH 7.4 containing 0.25 M sucrose solution and the liver was rapidly homogenized with ice-cold TEA buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at $10000 \times g$ for 10 min. The resulting supernatant was then centrifuged at $86000 \times g$ for 90 min to separate total Ms and soluble fractions. The former was resuspended in 1.15 M sucrose, and 10 ml of this suspension was placed beneath a discontinuous sucrose density gradient constructed by layering 8 ml of 0.86 M, 5 ml of 0.6 M, and 8 ml of 0.25 M sucrose containing 1 mM EDTA, pH 7.4. The centrifugation was performed at $63000 \times g$ for 210 min in a Beckman SW-27 rotor to obtain three Golgi subfractions, namely Golgi light, intermediate and heavy (Gl, Gi, and Gh), a smooth microsome fraction (SER), and a rough microsome fraction (RER). The individual fractions were collected by aspirating with a pipette from top to bottom of the tubes, and diluted with the same buffer, treated by freezing and thawing (F&T) repeated 10 times, and frozen at −80 °C until the determination of radioactivity. The ^{3}H and ^{14}C-radioactivity were determined by a liquid scintillation counter (Aloka LSC-900) after specimins were combusted and recovered as ^3H_2O and $^{14}CO_2$ in an automatic sample combustion system (Aloka ASC-112).

Extraction and Gel Filtration of ^{3}H and ^{14}C Radioactive Materials from

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Ms Sixty min after the injection, Ms was subjected to F&T 10 times in the presence of protease inhibitors (1 μM of 4-amidinophenyl-methansulfonyl fluoride (APMSF), leupeptin and pepstatin, respectively), and centrifuged at 105000 × g for 1.5 h. The supernatant was applied to a column of Sephadex G-150 (2 × 95 cm) equilibrated with 10 mM phosphate buffer, pH 7.4, and the recovery of radioactivity was 15%. The resulting residue after F&T was solubilized with Triton X-100 (1%), and centrifuged at 105000 × g for 2 h. Eighty five percent of the radioactivity in Ms residue was solubilized. The supernatant was subjected to a column chromatography under the same conditions except that the buffer contained Triton X-100 (1%). Blue dextran (void volume), rat Hb (68 kDa) and dinitrophenyl-ala (elution volume) were used as markers.

Separation and Determination of Bilirubin by Means of High Performance Liquid Chromatography (HPLC) Analysis of bilirubin extracted from the liver cell organelles was performed by HPLC after Yamaguchi *et al.*[10] Bilirubin was extracted with methanol/chloroform (1 : 2) 3 times after 10 times F&T repetitions of Golgi and SER subfractions, then analyzed using Waters ALC/GPC-244 apparatus. Samples (20 μl) were injected into a reverse phase column μBondapak C_{18} (3.9 mm × 30 cm, Waters Associates) with a jacket kept at 40 °C. After heme metabolites other than bilirubin were eluted by 100% methanol/5% acetic acid (70 : 30, v/v) at 1 ml/min flow rate, bilirubin IX α was eluted by 100% methanol at 32.4 min after the injection (at the same flow rate). A detector was set at 436 nm for the analysis.

Electron Microscope Radioautography Aliquots of the subcellular fractions isolated 20 min after injection of 72.0 × 10^6 cpm of [^{125}I]Hb–Hp (0.4 mg as Hb/100 g of body weight) were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 3 h at 4 °C. After washing, small aliquots of the fractions were postfixed by 1% OsO_4 in the same buffer for 2 h, dehydrated and embedded in Epon 812. Radioautography was carried out according to the method of Mizuhira and Futaesaku[11] with slight modifications. Thin sections were cut on an Sorval Porter-Blum ultramicrotome and the sections were picked up on collodion coated copper grids and stained with 2% uranyl acetate in 5% isopropranol for 20 min. They were then vacuum-coated with carbon and a monolayer or Ilford L-4 emulsion was applied. After 1 week, the radioautographs were developed with phenidoneascorbic acids.[12] The developed sections were recoated with carbon in vacuum and observed under a JEOL-100S electron microscope.

Chemicals δ-Amino[2,3-^{3}H]laevulinic acid L-(U-^{14}C)leucine was purchased from Commissariat a l'Energie Atomique, Na^{125}I from New England Nuclear. Other chemicals were of analytical grade, and doubly distilled water was used.

Results and Discussion

Dose Effect of [^{3}H-heme, ^{14}C-globin]Hb–Hp upon Hepatic Uptake

When administered in an amount of radioactivity Hb–Hp of 0.5 mg as Hb/100 g of body weight, both redioactivities attained maxima at 60 min after administration. The half-clearance time was 10 min. Hepatic uptake increased with time up to 40 min at both radioactivities. The ratios of ^{3}H to ^{14}C at latter times in both low and high doses (0.1 and 0.5 mg as Hb) increased with time, showing that the rate of globin metabolism is faster than that of heme metabolism (Fig. 1). There was no significant difference between the two doses in percentage of uptake, the ratio of [^{3}H]bilirubin derivatives derived from [^{3}H-heme]Hb–Hp in bile or subcellular distribution (data not shown) except a retardation of Hb–Hp metabolism. Since a 5-fold increase in injected dose does not disturb the Hb–Hp metabolism, the amount of 0.5 mg as Hb/100 g of body weight was used in the present investigation.

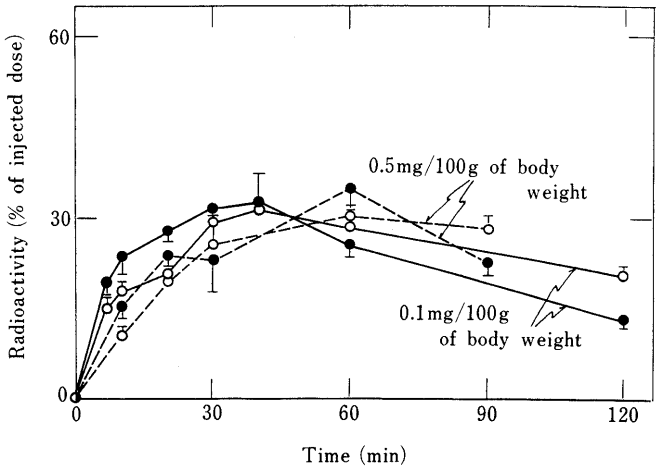

Fig. 1. Time Course of Hepatic Uptake of [^{3}H-heme, ^{14}C-globin]-Hb-Hp

After intravenous administration of [^{3}H-heme, ^{14}C-globin]Hb–Hp (0.1 or 0.5 mg as Hb/100 g of body weight) to rats, liver was removed and homogenized, and total ^{3}H (○) and ^{14}C (●) radioactivity was determined by measuring radioactivity in an aliquot of the homogenate. The ^{3}H and ^{14}C radioactivity was determined as described in Materials and Methods. The total radioactivity was expressed as a percentage of the injected dose. Each value is the mean ± S.D. of three experiments.

Time Course of Uptake of [^{3}H-heme, ^{14}C-globin]Hb–Hp into Golgi and Microsomal Subfractions

To examine the

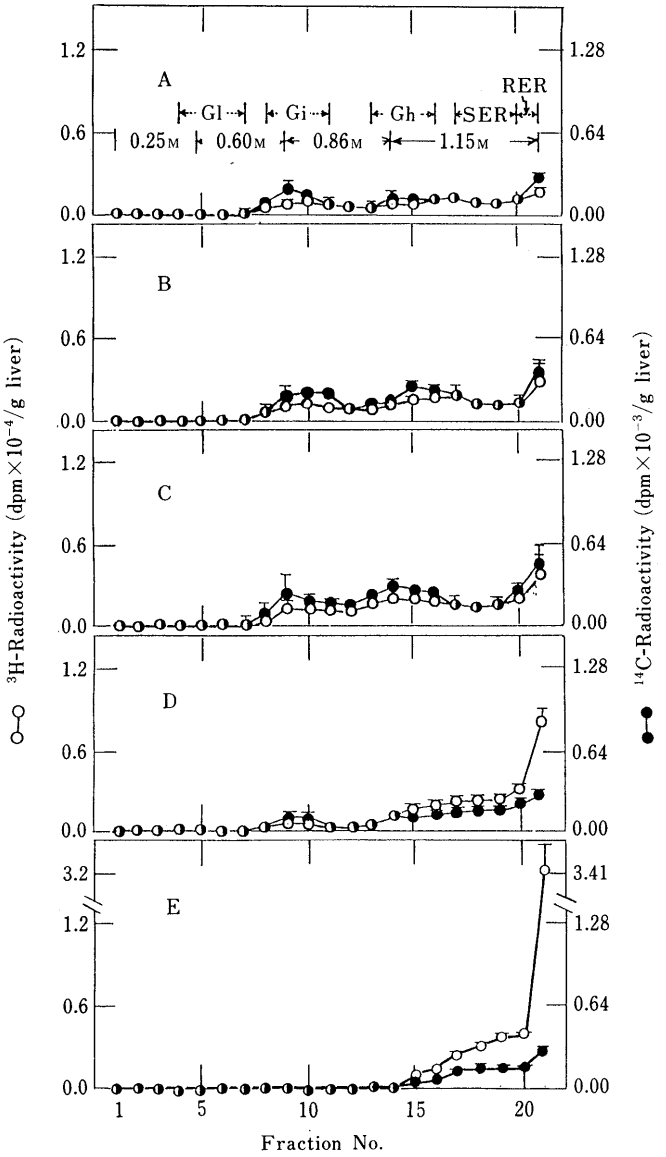

Fig. 2. Distribution of ^{3}H and ^{14}C Radioactivity in Individual Golgi and ER Subfractions as a Function of Time after Injection of [^{3}H-heme, ^{14}C-globin]Hb–Hp

Each point is the mean ± S.D. of three separate experiments. A, 10 min; B, 20 min; C, 30 min; D, 60 min; E, 90 min.

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distribution of endosomes taken up Hb–Hp and heme decomposition site in microsome subfraction, the amount of the radioactivity as well as the ratio of ^{3}H to ^{14}C activity (Fig. 2) were compared in Golgi and ER subfractions. Fraction number 4 to 7, 8 to 11, and 13 to 16 corresponded to Gl, Gi and Gh subfractions, respectively. Residual load zone (No. 17 to 20) and residual pellet (No. 21) corresponded to SER and RER subfraction, respectively. Specific enzyme activities of galactosyl transferase in Gl, Gi and Gh subfractions were approx. 183, 285 and 69 nmol galactose transferred/h/mg protein, and of 5′-nucleotidase were 6, 10 and 15 mol/h/mg protein, respectively. At 10 to 30 min, ^{3}H and ^{14}C radioactivity showed bimodal distribution in which most of the total radioactivities in Ms simultaneously localized in two fractions, Gi fraction (No. 8 to 11) and Gh fraction (No. 13 to 16). The ratio of ^{3}H to ^{14}C radioactivity showed the same value as that in the intact doubly labeled Hb–Hp up to 30 min postinjection. Shortly after [^{3}H-heme, ^{14}C-globin]Hb–Hp administration, Gi fraction enriched in galactosyl transferase was at the highest level of uptake (as compared not with dpm/g liver but dpm/mg of protein in each subfraction). On the other hand, the radioactivities in both SER (fraction No. 18 to 20) and RER (fraction No. 21) were relatively low, showing that endosomes taken up Hb–Hp initially and simultaneously distributes in Gi and Gh fraction. Thereafter, ^{3}H and ^{14}C radioactivity apparently shifted from these Golgi subfractions to SER and RER fractions. It is noteworthy that the increase in the ratio of ^{3}H to ^{14}C ratio in SER and RER fractions was more evident than that in other fractions at 60 and 90 min postinjection, suggesting that the heme or heme metabolites accumulate in organelles distributed in SER and RER fractions. This phenomenon is comparable to the distribution of heme degradation enzyme, heme oxygenase which distributes in Ms[12] and Golgi[13] fractions. However, since the ratio of $^{3}H/^{14}C$ fraction of SER and RER resembles that of Mt–Ly fraction[3] and lysosomal marker enzyme activity was observed in Ms,[2] a part of radioactivities may reflect the metabolism in Mt–Ly fraction.

Gel Filtration of ^{3}H and ^{14}C Radioactive Materials from Ms To examine the dissociation site of Hb–Hp into 82 kDa subunits, ^{3}H and ^{14}C radioactive materials from Ms 60 min postinjection were subjected to gel filtration (Fig. 3). Approximately 15% of the metabolites from Ms were extracted by F&T, which is parallel with the recovery of lysosomal marker enzymes in Ms. Hb–Hp metabolites in the extract are composed of Hb–Hp, 82 kDa subunits and Hb metabolites at a ratio of 1 : 6 : 13 (Fig. 3A). The heme metabolite extracted by F&T of Ms was identified as

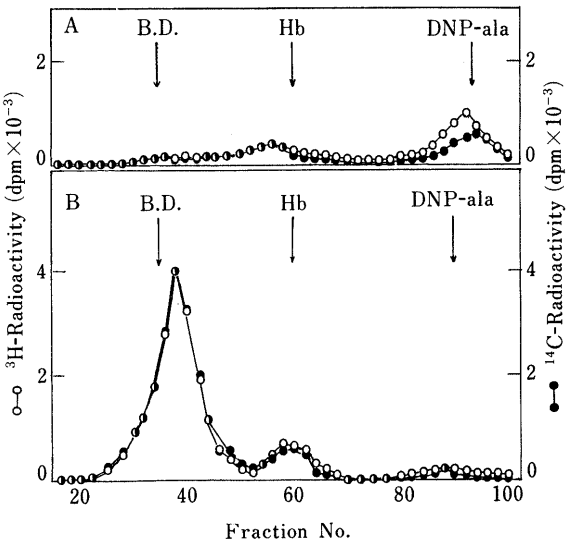

Fig. 3. Gel Filtration of ^{3}H and ^{14}C Radioactive Materials Extracted by Freezing and Thawing (A), and Solubilized by Triton X-100 from Ms Residue after Freezing and Thawing (B)

At 60 min after intravenous injection of [^{3}H-heme, ^{14}C-globin]Hb–Hp (0.5 mg as Hb/100 g of body weight) to rats, liver was removed and homogenized. The experiment was carried out as described in Materials and Methods. ○ and ● show ^{3}H and ^{14}C radioactivity, respectively. B.D., blue dextran.

TABLE I. ^{3}H Radioactivity of Heme Metabolites Separated by HPLC after Extraction from Golgi, Smooth and Rough ER Subfractions

Subfraction	Total radioactivity (dpm)	[^{3}H]Bilirubin (dpm)	Relative amount of bilirubin (% of total)
Gl	1666	N.D.	N.D.
Gi	2042	N.D.	N.D.
Gh	2116	357	16.9
SER and RER	77031	770	1.0

^{3}H radioactive materials extracted from Golgi and ER subfractions were analyzed by HPLC 30 min after administration of doubly labeled Hb-Hp (0.5 mg as Hb/100 g of body weight) at described in Materials and Methods. N.D. means not detected.

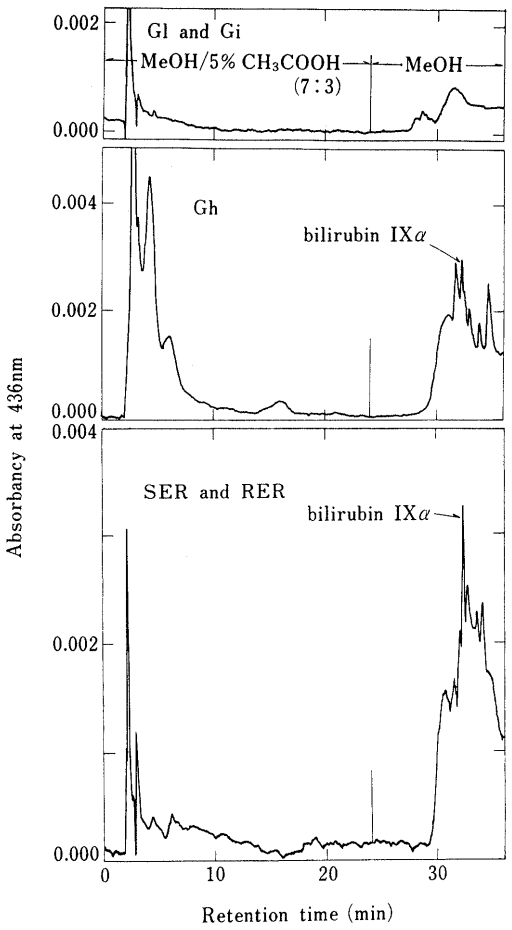

Fig. 4. High-Performance Liquid Chromatograms of Bilirubin IXα Extracted from Golgi and ER Subfractions

The preparation of samples and chromatographic conditions were as described in Materials and Methods. The retention time of bilirubin IXα standard was 32.2 min. The typical data are shown.

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[^{3}H]bilirubin (Table I). At 5 min after the injection, only Hb–Hp was recovered from Ms by F&T, or by Triton X-100 treatment. The recovery of each fraction was 10 and 85% (data not shown). Regarding the resulting residual pellet, most of radioactivity in the residue (85%) could be solubilized with Triton X-100. On the contrary, the ratio of Hb–Hp/82 kDa/Hb metabolite of these extracts was 40 : 8 : 1 (Fig. 3B). Taking into consideration the F&T treatment: 1) 70 to 80% of Hb–Hp metabolites was extracted from Mt–Ly fraction 60 min postinjection 2) since microsomes are resistant to F&T and only 10% of Golgi vesicles are disrupted by this treatment,[9] most ^{3}H and ^{14}C radioactive materials extracted with F&T (Fig. 3A) of Ms seem to be Hb–Hp metabolites derived from the metabolism in Ly. On the other hand, radioactive materials solubilized by detergent from Ms residue (Fig. 3B) seem to be derived from microsomes containing endosomes and Golgi vesicles. In addition, 82 kDa subunits were solubilized with both treatments. Therefore, it is suggested that after Hb–Hp is dissociated into two 82 kDa subunits in organelles of Ms, this subunit is degraded in Mt–Ly fraction.

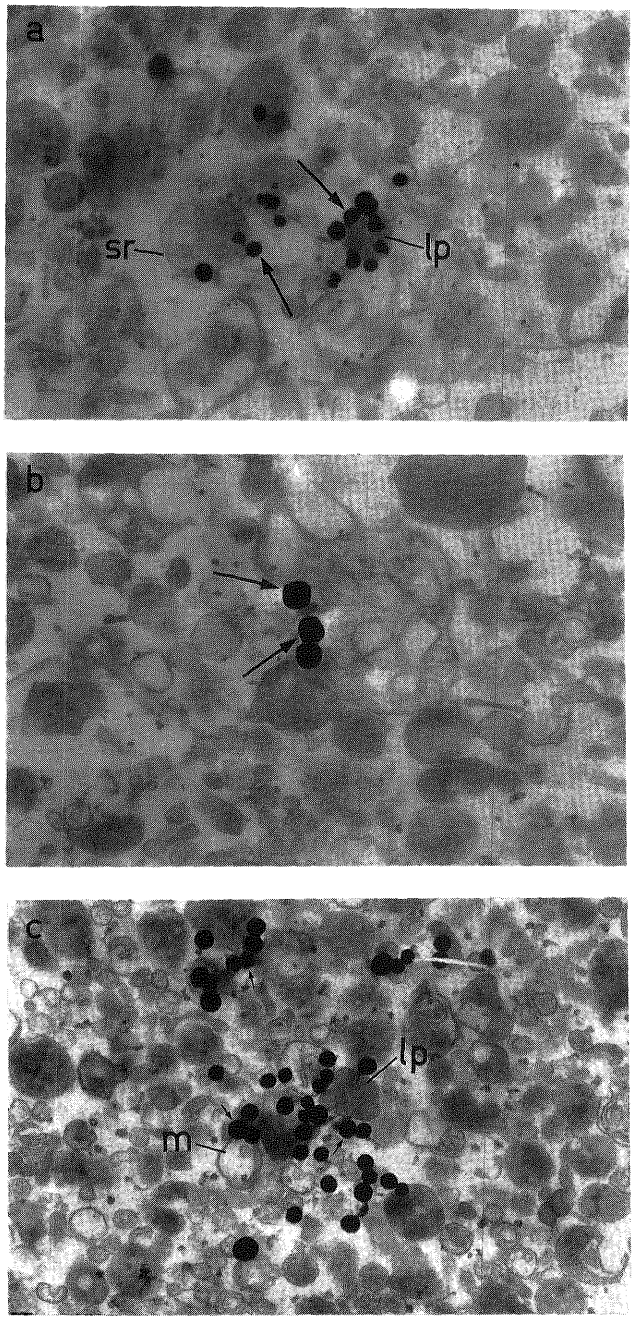

Fig. 5. Electron Microscope Radioautographs of Golgi Subfractions Isolated 20 min after the Injection of 72×10^6 cpm of [^{125}I]Hb–Hp

The exposure period was for 7 d. (a) Gl (× 40000); (b) Gl (× 40000); (c) Gi (× 40000). In a, silver grains are mainly found in the periphery (arrows) of particles and the structure with a signet-ring appearance (sr) which resembles MVB or CURL. In b, some grains are seen at a particular point of a large vacuole (arrows) with small particles. In c, there are several silver grains (small arrows) associated with membranous elements (m) devoid of lipoprotein particles as well as vesicles filled with VLDL-like contents (lp).

Analysis of Heme Metabolites in Golgi and ER Subfractions by HPLC Thirty min after injection of [^{3}H-heme, ^{14}C-globin] Hb–Hp (0.5 mg as Hb/100 g of body weight), heme or heme metabolites were extracted from Golgi and ER subfractions, and separated by HPLC (Fig. 4). ^{3}H radioactive materials from these fractions were extracted with methanol/chloroform (1 : 2, v/v) for biliverdin and bilirubin after F&T, and acidic ether was used to liberate heme from Hb or a heme binding protein, except for globin after ten repetitions of F&T. No heme metabolites were observed except for intact [^{3}H]heme at 10 min.[3] At 30 min, more than 90% of the ^{3}H radioactive materials was identified as intact heme in each fraction, whereas a ratio of [^{3}H]bilirubin to total radioactivity in Gh was higher than that of other fractions (Table I). As shown in Fig. 4, Gh and both ER fractions contained a significant amount of endogenous bilirubin. The results were as follows: 1) ^{3}H radioactive material was liberated from organelles sensitive to F&T. 2) Since a relatively high amount of [^{3}H]-bilirubin was observed in Mt–Ly fraction,[3] ^{3}H radioactive bilirubin may originate in heme metabolite from Ly. However, Tenhunen[12] and Hino *et al.*[13] previously reported that heme oxygenase (which converts heme to biliverdin) distributes in Ms and Golgi fractions. Accordingly, bilirubin from Hb–Hp may accumulate in Mt–Ly fraction after the heme degradation at Golgi and/or microsome.

Radioautography of Golgi Subcellular Fractions Hb is composed of porphyrin IX, iron and globin moiety, which are destined to be processed separately. Accordingly, organelles responsible for the metabolism must be obligatory for multi-functions, the sorting of these components and recycling of Hb–Hp receptor. To investigate the morphology of organelles responsible for Hb–Hp metabolism, electron microscope radioautography was performed on Golgi subfractions using [^{125}I]Hb–Hp (Fig. 5). Electron micro-

Table II. Grain Distribution in Golgi Subfractions

Fraction	Number of grains	Location	% distribution
Gl	452	Over membrane	71.9
		Inside vesicles	23.7
		Outside vesicles	4.4
Gi	479	Over membrane	69.3
		Inside vesicles	27.1
		Outside vesicles	3.6
Gh	269	Flattened saccules	38.7
		Vesicles	59.1
		Outside structures	2.2

Radioautographic grains shown in Fig. 4 were counted as appearing over membrane if any part of the grains touched the membrane. If not, they were ascribed to the inside vesicle depending on their location. In analyzing the Gh fraction, the distribution of flattened saccules was determined.

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scope analysis demonstrated that silver grains in Golgi fraction were always found over lipoprotein-filled structures and over their periphery. Silver grains in G1 subfraction were mainly found in the periphery of VLDL-like particles and several grains were observed in signet-ring structures like appendage MVB or CURL (Fig. 5a).[14,15] And some grains occasionally appeared clustered over one pole of a large vesicle packed with empty vacuoles (Fig. 5b). In Gi subfraction, the silver grains were always observed in association with VLDL-containing vesicles and tubules, and often seen in a vacuole (Fig. 5c). In Gh subfraction, the grains were found over both flattened saccules (data not shown). A preferential localization of grains to the membrane of Golgi elements was also suggested by quantitative analysis (Table II). These phenomena may be associated with MVB or CURL-like vesicles rather than heterogenous endosomes since their characteristic structures are still maintained even after Percoll fractionation of rat liver homogenate[16] as shown in Fig. 5a.

On the basis of these results, it is suggested that Hb–Hp taken up *via* RME is symmetrically dissociated into two 82 kDa subunits in organelles of Ms, and that MVB or CURL-like vesicles are associated with Hb–Hp metabolism.

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