42. Studies on Hemoglobin Metabolism. VII

*Intrahepatocellular Site of the Incorporation of Heme and Globin Moiety of Hemoglobin-Haptoglobin after Intravenous Administration to Rats**

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As has been reported previously, hemoglobin-haptoglobin (Hb-Hp) formed *in vivo* by intravascular hemolysis is incorporated into the liver parenchymal cells through the receptor specific for the molecule.¹⁾

The present investigation was designed to clarify the intracellular site of incorporation of heme and globin moiety of Hb-Hp in liver cells after intravenous administration of [³H-heme,¹⁴C-globin]Hb-Hp to rats.

Materials and methods. [³H-heme,¹⁴C-globin] Hb was synthesized by incubating reticulocytes of newborn rats with 3 mCi of δ -amino [³H-2, 3] levulinic acid (CEA, 29 Ci/mmol) and 0.1 mCi of L-[U-¹⁴C] leucine as reported previously.²⁾ Specific ³H- and ¹⁴C-radioactivities of the labeled Hb were 184.02×10^4 dpm/mg and 21.73×10^4 dpm/mg, respectively. [³H-heme,¹⁴C-globin]Hb-Hp was prepared by the addition of [³H-heme,¹⁴C-globin] Hb to a partially purified rat Hp. Male Wistar rats weighing 150 to 200 g were fasted overnight, and [³H-heme, ¹⁴C-globin] Hb-Hp (0.1 mg as Hb/100 g of body weight) was injected intravenously *via* portal vein. In order to determine organ distribution of the doubly-labeled Hb-Hp at indicated times postinjection, animals were killed by exsanguination by perfusion with approximately 50 ml of 0.25 M sucrose through abdominal vein. The liver, kidney, and spleen were removed, washed and homogenized with 2.5 mM triethanolamine-acetate buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA. Aliquots of these samples were taken for counting of radioactivities. Subcellular fractionation by means of differential centrifugation was carried out by the modified method of Sawant *et al.*³⁾ The liver was minced and homogenized in 4 volumes of 2.5 mM triethanolamine-acetate buffer, pH 7.4, contain-

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ing 0.25 M sucrose and 1 mM EDTA, using a glass homogenizer with Teflon pestle. The homogenate was spun at $750\times g$ for 10 min to remove nuclei and cell debris, and the crude mitochondrial and lysosomal fractions were obtained by centrifugation of the post-nuclear supernatant at $3,300\times g$ for 10 min and $10,000\times g$ for 10 min, respectively. The resulting supernatant was centrifuged at $86,000\times g$ for 60 min, and the pellet and supernatant obtained were referred to

Table I. Subcellular distribution of ^3H - and ^{14}C -radioactivity at different times after administration of [^3H -heme, ^{14}C -globin] Hb-Hp to rats

Time (min)	Homogenate		Mitochondrial fraction			
	% of injected dose		% of homogenate			
	^3H	^{14}C	^3H	^{14}C		
5	19.8±3.3	26.5±4.8	10.2±2.1	9.5±1.6		
10	23.6±4.5	31.8±2.8	10.1±1.4	9.7±2.2		
20	34.9±3.6	40.2±1.6	10.7±0.3	9.4±0.3		
30	38.4±0.4	41.0±9.9	12.2±0.2	10.9±0.3		
40	42.7±5.8	43.4±2.7	10.6±0.1	10.4±0.3		
60	40.1±2.0	36.8±2.3	9.3±0.1	8.9±1.1		
120	27.6±2.4	16.0±0.6	22.9±0.1	18.8±0.7		

Time (min)	Lysosomal fraction		Microsome fraction		Cytosol fraction	
	% of homogenate					
	^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
5	13.2±0.5	11.7±1.7	40.6±7.6	40.3±4.9	16.5±2.7	13.0±2.6
10	15.4±3.3	15.0±3.7	49.7±4.9	46.6±0.2	5.8±1.7	7.7±2.5
20	19.6±0.4	16.8±0.5	44.9±7.7	40.6±0.7	11.5±2.3	12.4±0.2
30	18.2±1.3	15.3±0.2	31.9±3.8	35.4±3.2	21.6±2.8	20.0±3.6
40	14.0±0.4	15.1±0.9	31.7±0.9	25.6±4.9	19.2±2.8	19.7±1.8
60	17.3±2.3	14.5±0.3	30.0±4.3	32.8±1.7	16.0±0.5	11.1±4.6
120	14.5±1.7	14.1±1.5	27.5±3.5	35.1±1.2	18.7±2.4	11.7±2.8

Each value is the mean $\pm$ standard deviation of 3 experiments.

as microsome and cytosol fractions, respectively. Another procedure employed for separation of subcellular organelles of liver cells was Percoll density-gradient centrifugation.⁴⁾ Postnuclear supernatant of liver homogenate was mixed with Percoll density-gradient medium in 0.25 M sucrose to give a density of 1.082 g/ml, and then centrifuged at $105,000\times g$ for 20 min. The gradient fractions were collected from centrifuge tubes to obtain 20 fractions. Golgi subfractions were isolated according to Ehrenreich *et al.*⁵⁾ with slight modification. Microsome fraction, which corresponded to the low-density fraction⁶⁾

in Percoll density-gradient centrifugation, was suspended in 10 ml of 1.15 M sucrose and was placed beneath a discontinuous sucrose gradient constructed by layering 8 ml of 0.86 M, 8 ml of 0.6 M, and 5 ml of 0.25 M sucrose. The centrifugation was performed at $63,500 \times g$ for 210 min to obtain three Golgi subfractions (Golgi light (Gl), Golgi intermediate (Gi), and Golgi heavy (Gh)), smooth endoplasmic reticulum (SER) (residual road zone), and rough endoplasmic reticulum (RER) (residual pellet). The subcellular fractions were treated by repeated freezing and thawing at least 5 times, and marker enzyme activities were determined as estimation of the purity of the fractions as described previously.⁷⁾

Results and discussion. After injection *via* portal vein, [³H-heme, ¹⁴C-globin]Hb-Hp was cleared from circulation with a half-

Fig. 1. Distribution of ³H- and ¹⁴C-radioactivity in fractions obtained by Percoll density-gradient centrifugation of postnuclear supernatant of liver homogenate at different times after administration [³H-heme, ¹⁴C-globin]Hb-Hp to rats.

clearance time of 5 min, and incorporated exclusively into the liver, and the radioactivity in liver attained a maximum at 40 min after administration and declined thereafter. At 30 min after administration, hepatic uptake of ³H-radioactivity was 38%, with less than 5% recoverable from kidney and less than 3% recoverable from spleen.

The uptake of ^{14}C -radioactivity in these organs was similar to that of ^3H -radioactivity. Subcellular distribution of ^3H - and ^{14}C -radioactivity in the liver was determined after administration of the doubly-labeled Hb-Hp to rats, and relatively higher specific radioactivity was observed in microsome and lysosome fraction at 20 to 40 min after injection. From 5 to 30 min after injection, 35 to 50% of the total radioactivity in the homogenate was recovered in microsome fraction (Table I). While the specific radioactivities in cytosol showed much lower level than those in other fractions throughout the investigation. It was also shown that after centrifugation at $750\times g$ for 10 min, more than 80% of ^3H - and ^{14}C -radioactivity in the original homogenate were recovered in the supernatant (Table I), and this supernatant was further subjected to Percoll density-gradient centrifugation. Distribution of marker enzyme activities in

Fig. 2. Time course of uptake of ^3H - and ^{14}C -radioactivity into liver homogenate, microsome fraction, and Golgi fraction after administration of [^3H -heme, ^{14}C -globin] Hb-Hp to rats. Each point is the mean $\pm$ standard deviation of three experiments.

fractions after Percoll density-gradient centrifugation of postnuclear supernatant from rat liver homogenate was coincided well with the previous communication.⁷⁾ Time course of intracellular distribution of the radioactivity after the doubly-labeled Hb-Hp was investigated by means of Percoll density-gradient centrifugation (Fig. 1). At 5 min after injection, ^3H - and ^{14}C -radioactivities were demonstrated in fractions of low density (1.03–1.05 g/ml), and then the distribution of the radioactivities throughout the density-gradient shifted

progressively from the particles of low density to the fractions of higher density (1.06–1.12 g. ml) representing two major radioactive peaks. At later times of the investigation, both ^3H - and ^{14}C -radioactivities were declined representing bimodal distribution. It is noteworthy that the increase in the ratio of ^3H - to ^{14}C -radioactivity is prominent in fractions of higher density than that in fractions of low density at later times postinjection (Fig. 1). This increase in the $^3\text{H}/^{14}\text{C}$ ratio was also observed in whole homogenate at later times after administration of the doubly-labeled Hb-Hp (data not shown). The particles of low density in Percoll density-gradient centrifugation were corresponded to microsome fraction in differential centrifugation.⁶⁾ In the subsequent study, therefore, the microsome frac-

Fig. 3. Time course of association of ^3H - and ^{14}C -radioactivity with individual Golgi subfractions following injection of [^3H -heme, ^{14}C -globin]Hb-Hp to rats. Each point is the mean $\pm$ standard deviation of three experiments.

tion was separated into Golgi subfractions and both smooth and rough endoplasmic reticulum after Ehrenreich *et al.*⁵⁾ Time course of uptake of ^3H - and ^{14}C -radioactivity into total Golgi subfraction, microsome fraction, and homogenate was shown in Fig. 2. Incorporation of the both radioactivity into liver homogenate reached a maximum at 40 min after injection of the doubly-labeled Hb-Hp. In microsome

fraction, uptake of radioactivity was at maximum by an earlier time, 10 min postinjection. Prominent peaks of ^3H - and ^{14}C -radioactivity in total Golgi subfraction occurred at 7 and 10 min, respectively, after injection. The specific radioactivity of microsome fraction was approximately 3 folds of that of homogenate. In total Golgi subfraction, the specific radioactivity was approximately 10 times higher than that of microsome fraction. From 3 to 20 min after injection of the doubly-labeled Hb-Hp, approximately 20 to 40% of ^3H -radioactivity and 35 to 50% of ^{14}C -radioactivity in microsome fraction were distributed in total Golgi subfraction (data not shown). Time course of uptake of the radioactivities into three Golgi subfractions were shown in Fig. 3. Specific radioactivity reached near a maximum at 5 to 7 min after injection of the doubly-labeled Hb-Hp in Golgi heavy fraction (Gh). Uptake of the radioactivity in Golgi intermediate fraction (Gi) attained a peak at 10 to 15 min, and in Golgi light fraction (Gl) at 15 to 20 min, respectively. The result indicates that [^3H -heme, ^{14}C -globin]Hb-Hp is incorporated first into Gh, then Gi, and finally Gl fraction.

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