97. Histological Studies on Hemoglobin Degradation. I

*Radioautographic Study in Rat Liver and Kidney**

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Introduction. It has been known that hemoglobin (Hb) released into the blood stream combines with haptoglobin (Hp), and this Hb-Hp complex is taken up and disintegrated by liver.¹⁾⁻¹²⁾ However, it is not clear how Hb-Hp complex is processed in the liver. Several studies have reported that Hb-Hp complex is taken up and degraded by the reticuloendothelial cells including Kupffer cells.^{2),3),6)-8)} On the other hand, it was also proposed that the complex is degraded selectively in hepatocytes.^{1),4),5),9)-12)} In these studies, however, it should be noted that no direct evidence based on histological survey is available, and ⁵⁹Fe-labeled Hb was frequently used.⁹⁾⁻¹²⁾ The removal of Fe from Hb occurs in liver shortly after the administration of Hb, and Fe binds with its binding proteins or incorporated into hepatic ferroproteins such as ferritin or cytochromes. Therefore, ⁵⁹Fe-labeled Hb is not suitable for the study of Hb degradation in liver. The above discrepancy may be due to these problems.

In the series of our studies, the fate of Hb in the liver was directly investigated *in situ* by radioautography using ³H-labeled Hb which was synthesized *in vivo* with extremely high specific radioactivity. This communication points out that the findings using rat Hb may be misleading because of its very low solubility in physiological pH range.

Materials and methods. ³H-Hb of rat was synthesized by incubating the reticulocytes of newborn rats with 1.0 mCi of ³H-2-glycine and ³H-4,5-leucine (20 and 30 Ci/mmol, NEN). It has been known that fetal Hb is not found in rat erythrocytes after 16 days of gestation.¹³⁾⁻¹⁴⁾ The specific radioactivity of Hb was 2.0×10^7 dpm/mg.

Male Wistar rats (300 g) were used in this study. A small and large amount (3.7 and 12.6 mg) of ³H-Hb which corresponds to about

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0.5 and 2.0-fold serum Hb binding capacity, respectively, was injected into the tail vein of the rat. Fifteen min after the injection, liver and kidney were removed quickly, cut into small pieces and immersed in Karnovsky's fixative¹⁵⁾ for 2 hrs at 4°C. The tissues were then washed in 10% buffered sucrose, postfixed in 1% OsO₄ for 1 hr, dehydrated in graded series of cold ethanol and embedded in Epon 812. Semithin sections (0.5 μ m thick) were made for light microscopic radioautography using Sakura NR-M2 nuclear emulsion (Konishiroku Photo Ind., Tokyo). After 8 weeks, each section was developed in Konidol-X (Konishiroku Photo Ind., Tokyo) and stained with 1% toluidine blue for microscopic analysis.

Disc electrophoresis was performed using 7.5% polyacrylamide gels. For the detection of the radioactivity, the gel was sliced in 1.2 mm thick by a gel slicer and each section was incubated in 1.0 ml of Protosol (NEN)/H₂O, 9:1 (v/v) for 2 hrs at 60°C, neutralized with acetic acid, and then counted in a liquid scintillation counter using toluene scintillator. The radioactivities of Hb, fixative and alcohol were determined in toluene·Triton X-100 scintillator (2:1).

Table I. Retainment of radioactivity in liver tissue during fixation and dehydration

Experiment	Count in tissue	Fixative	Washing buffer	Osmium	Ethanol				Elution of radioactivity
					65%	75%	85%	100%	
			(dpm)						(%)
1	480,000	18,500	1,550	0	0	0	0	0	4.2
2	480,000	12,600	2,420	590	0	0	0	0	3.3
3	406,000	18,500	1,800	9	0	0	0	0	5.0

Rats were injected intravenously with 3.7 mg of ³H-hemoglobin. Fifteen min after the injection, liver slices (100-120 mg) were prepared.

Results and discussion. Table I shows the radioactivity which is lost during the fixation and dehydration of tissue blocks. The radioactivities in glutaraldehyde solution and the washing buffer were only 3-5% of that in the tissue, and no radioactivity was found in the alcohol series. Therefore, nearly all of the radioactivity remains within blocks during the preparation.

To examine whether injected Hb combines with Hp *in vivo*, disc electrophoresis of the serum was performed after the administration of a small amount of ³H-Hb, being about 50% of Hb-binding capacity in rat serum. As shown in Fig. 1, 15 min after the injection, all of the radioactivity was recovered as Hb-Hp complex, indicating that no free Hb was present in the serum in this situation. In contrast, free Hb was observed in serum when a large amount of Hb was

Fig. 1. Polyacrylamide gel electrophoresis of serum of rat after the administration of ^3H -hemoglobin. A rat was injected intravenously with 3.0 mg of ^3H -hemoglobin. Fifteen min after the injection, 0.1 ml of serum was applied to the gel, and the electrophoresis was performed as described under Materials and methods. For the electrophoresis of ^3H -hemoglobin, 0.1 mg was used. Arrow indicates the mobility of standard hemoglobin-haptoglobin complex. ●-●; serum, ○-○; ^3H -hemoglobin.

administered.

The radioautograms of liver after the administration of ^3H -Hb are shown in Fig. 2, a and b. The silver grains were located in periphery of parenchymal cells, showing Hb that has just been taken up by the hepatocytes. A regional difference in the uptake of ^3H -Hb by hepatocytes was noted. Some groups of hepatocytes, being composed of several cells, were highly labeled, and such groups were scattered in the liver with no definite localization. The grains were also observed in Kupffer cells and endothelial cells. No difference was observed in the uptake of Hb by the liver cells between the administration of small and large amount of Hb.

Fig. 3, a and b show the radioautograms of the kidney. When a small amount of Hb was injected, no silver grain was observed in any of the kidney cells. In contrast, with large dose of Hb, silver grains were observed in proximal tubule cells, but not in distal and collecting tubule cells. It appears that free Hb was passed through the glomerulus due to small molecular size, and taken up by the epithelial cells of proximal tubules. It has been reported that Hb is also metabolized by kidney in severe hemolysis.¹⁶⁾⁻¹⁹⁾ In kidney, free Hb seems to be selectively reabsorbed by proximal tubule cells.

The present findings are consistent with the previous reports,^{1), 4), 5), 9)-12)} which have been shown that Hb-Hp complex was

Fig. 2. Radioautogram of liver 15 min after the administration of ³H-hemoglobin. Rats were injected with ³H-hemoglobin of 0.5 (a) and 2.0 (b)-fold serum hemoglobin-binding capacity. Bar; 5 μ m.

taken up by liver, but not by kidney. However, any conclusive finding was not obtained to clarify whether Hb-Hp complex is taken up by hepatocytes or Kupffer cells. It seems that the complex is taken up by hepatocytes, since the uptake of ³H-Hb by hepatocytes was similar in low and high dose of Hb, whereas the uptake by Kupffer cells increased in high dose. Kupffer cells may uptake the free Hb.

The silver grains in Kupffer cells seem also to be due to the unusually low solubility of rat Hb under the physiological pH.²⁰⁾⁻²²⁾ The crystallization of rat Hb even in erythrocytes has been reported.²³⁾ It is also known that Hb-Hp complex of rat is easily crystallized.²⁴⁾ Such

Fig. 3. Radioautogram of kidney 15 min after the administration of ³H-hemoglobin. Rats were injected with ³H-hemoglobin of 0.5 (a) and 2.0 (b)-fold serum hemoglobin-binding capacity. Bar; 20 μ m.

a crystallized Hb-Hp complex may be recognized and taken up by the Kupffer cells as a foreign molecule. This characteristics of rat Hb may explain the selective uptake of Hb-Hp complex by Kupffer cells in the several reports using rat Hb.^(2),3),7) Furthermore, rat Hb shows marked molecular heterogeneity, in which 6 different molecules were observed.^(14),25) It seems possible that several Hp complexes of 6 Hbs are taken up by Kupffer cells. Thus, care must be taken for the study on the recognition mechanism of Hb by liver cells using rat Hb.

In the following communication,⁽²⁶⁾ the experiments will be reported using mouse, in which Hb has a high solubility in the physio-

logical pH as human Hb and no heterogeneity in Hb is observed. In the mouse, Hb-Hp complex is nearly selectively taken up by hepatocytes, and little uptake is observed in Kupffer cells, indicating that Hb-Hp complex is taken up and degraded by hepatic parenchymal cells.

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