THE RENAL HANDLING OF HEMOGLOBIN

II. Catabolism*, †

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(Received for publication 6 January 1969)

Upon exceeding the binding capacity of haptoglobin, plasma hemoglobin readily enters the kidney. A mechanism for the glomerular filtration of hemoglobin is proposed in the preceding paper (1). Morphologic (2, 3) and functional (4, 5) studies indicate that the filtered molecule is absorbed in the proximal tubule. Only when this absorptive capacity ($T_m$) is exceeded, will hemoglobin appear in the urine. Thus, free hemoglobin is handled by the nephron in a manner similar to glucose, phosphate, uric acid, amino acids, and probably other proteins.

The way in which the kidney handles absorbed hemoglobin has not been well elucidated. Most of the previous studies have been morphologic and have involved very large doses of frequently impure and sometimes heterologous hemoglobin. We have approached these problems by the administration of small doses of pure isotopically labeled hemoglobin to the haptoglobin depleted rat.

Materials and Methods

General Procedures.—All experiments were done using Caesarean-derived male rats of the Sprague-Dawley strain which weighed from 250 to 400 g. Animals for each experiment were of uniform age and were divided into experimental groups of approximately equal size. 1.0 ml injections were made into tail veins after the animals had been lightly anesthetized with ether.

Preparation of Radioactive Hemoglobins.—$^{59}$Fe rat hemoglobin was prepared as outlined by Keene and Jandl (7). $^{14}$C-labeled rat hemoglobins were prepared as follows. Reticulocytes were obtained by the removal of 30% of the blood volume from an iron-loaded rat on 3 successive days. On the 4th day, blood containing 50–70% reticulocytes was harvested. To these cells were added three parts of isologous heparinized plasma containing added glucose (2.0

* Presented in part at the 81st annual meeting of the Association of American Physicians, May, 1968.
† In conducting the research described in this paper, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences–National Research Council.
mg/ml) and uniformly labeled 14C-leucine or 2-14C-glycine. The mixture was incubated with gentle shaking at 37°C for 3 hr. The supernate was then removed and the cells washed once. Then the cells were incubated for an additional hr in unlabeled plasma, which served as a "chase."

Hemoglobin solutions were prepared as described in the previous report (1). The radioactive hemoglobins were then purified by passage through a column of Sephadex G-25. In this way 14C-labeled amino acid precursors were readily separated from the labeled hemoglobin. The hemoglobin solutions were prepared for injection by dilution with a mixture of one part isotonic phosphate buffer (pH 7.4) and two parts isotonic NaCl, to give a final hemoglobin concentration of 2 mg/ml. Methemoglobin was prepared by the addition of a 1.2 equivalent excess of K4Fe(CN)6 followed by dialysis against isotonic buffered saline. Cyanmethemoglobin was made from methemoglobin by the addition of a 1.2 equivalent excess of neutral cyanide, again followed by dialysis. Carboxyhemoglobin was prepared by gassing oxyhemoglobin with carbon monoxide.

Schedule of Injections.—Unless otherwise stated, all rats were depleted of haptoglobins by two successive injections of 10 mg of unlabeled hemoglobin 1 hr apart. 1 hr later, the test dose was given and consisted of 2 mg of a labeled hemoglobin. In some experiments, control animals were given saline instead of unlabeled hemoglobin and therefore had normal amounts of plasma haptoglobin, sufficient to bind totally the 2 mg test dose. Animals were sacrificed in groups of three, at varying times after the test dose. Urine was collected from rats by use of metabolic cages.

Measurement of Radioactivity.—

Iron-59: The liver, spleen, kidneys, and left femur were removed, rinsed in tap water, and blotted with gauze sponges before being weighed. Radioactivity measurements were made on approximately 1 g of liver, 1 kidney, 1 divided femur, 1 ml of whole blood, and 1 ml of plasma in a well type scintillation counter for a minimum of 10 min or 10,000 counts. Total body activity and urine activity were measured in a large well scintillation counter of high counting efficiency. Suitable corrections for counting geometry and for the natural decay of the isotope were made.

Carbon-14: In these experiments, only the plasma and kidneys were counted. Plasma activity was measured by counting 1 ml of a 1:100 dilution of plasma in distilled water on planchets with a low background gas flow counter. Kidney protein activity was measured as follows. The minced organ was homogenized in cold 10% trichloroacetic acid (TCA). After the protein precipitate was washed with 10% TCA, ethanol, and ethyl ether, it was dissolved in 0.2 N NaOH and an aliquot was counted. Parallel runs using a mixture of unlabeled kidney homogenate and 14C-labeled hemoglobin (prepared from 14C-leucine) indicated that about 90% of the protein activity was recovered.

14C-heme activity was measured in kidney tissue of rats given hemoglobin labeled with 2-14C-glycine. To the kidney homogenate about 80 mg of unlabeled hemoglobin was added as carrier. Crystalline hematin was then isolated from this mixture by the method of Labbe and Nishida (6). The hematin was dissolved in 0.2 ml of 0.1 N NaOH and diluted to 3.0 ml with water. 1.0 ml aliquots were counted. The total recovery of hematin (as measured by absorption at 370 mμ) was generally 30-50%. The total hematin activity per kidney was estimated from the recovered activity and the percent recovery.

Calculations: The total red cell 65Fe activity was calculated from the counts per ml of heparinized blood and the blood volume estimated from body weight by the data of Keene and Jandi (7). Total liver activity was estimated from the counts/g of liver tissue and the
total liver weight. The marrow activity was estimated from femur activity and the distribution of hemoglobin in the rat skeleton, as described previously (7). Organ activity was related to that of the infused hemoglobin. 14C-hematin activity in the kidneys was likewise related to the heme activity of the injected hemoglobin. In the following figures, the ordinate is the percentage of the total injected activity, detected in the kidneys.

RESULTS

 Fate of Hemoglobin Iron.—Haptoglobin depleted rats had a renal uptake amounting to 37 ± 3% of the test dose of 59Fe hemoglobin 1 hr after injection.

At this time, 96% of the plasma had been cleared of hemoglobin activity. The remaining activity was found primarily in the reticuloendothelial system in a distribution similar to that found by Keene and Jandl (7). The hemoglobin iron which was taken up by the kidneys disappeared very slowly. Animals were sacrificed at six intervals after the test dose. As shown in Fig. 1, half of the kidney activity remained after 37 days. Urine of four of these animals was collected continuously. The average cumulative 59Fe excretion at 25 days after injection was only 1.5 ± 0.4% of the test dose, as compared with 0.7 ± 0.4% in a control group which had not been depleted of haptoglobin. Total
body activity of the haptoglobin depleted group was, if anything, slightly in excess of the control group. The organ distributions of $^{59}$Fe in the haptoglobin-depleted and haptoglobin-intact rats 37 days after injection are shown in Table I. The total percentage of accountable iron was slightly higher in the haptoglobin depleted group than the haptoglobin intact group, although the difference is not significant ($P = 0.1$).

In a parallel experiment, the $^{59}$Fe hemoglobin dose was 10 mg instead of 2 mg. The initial 24 hr urine activity was 18 ± 5% of the test dose as compared to 0.5-0.2% in the low dose group. Otherwise the organ distribution of radioactivity and the rate of decline in kidney activity over 37 days was approximately the same as the low dose group.

Conversion of the test dose hemoglobin to methemoglobin, cyanmethemoglobin, or carboxyhemoglobin had no effect on the $^{59}$Fe activity of the kidneys removed 1 hr after injection into haptoglobin-depleted rats.

Fate of Hemoglobin Heme.—Hemoglobin was labeled both in the heme and in the globin by the incubation of rat reticulocytes with $^{14}$C-glycine. In a manner similar to the experiments described above, haptoglobin-depleted rats

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**TABLE I**

<table>
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<tr>
<th>Radioactivity</th>
<th>Red cells</th>
<th>Liver</th>
<th>Spleen</th>
<th>Marrow</th>
<th>Kidneys</th>
<th>Total</th>
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<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
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<td></td>
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<td>10.9</td>
<td>0.7</td>
<td>2.4</td>
<td>23.0</td>
<td>74.8</td>
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<td>19.0</td>
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<td>24.8</td>
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<td>19.3</td>
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<td>2.2</td>
<td>19.5</td>
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<td>8.1</td>
<td>0.5</td>
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<td>20.8</td>
<td>63.3</td>
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<td>Mean ± 1 SD</td>
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<td></td>
<td></td>
<td>71.1 ± 6.7</td>
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<td>2.2</td>
<td>1.1</td>
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<td>54.6</td>
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<td>Mean ± 1 SD</td>
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<td></td>
<td>62.1 ± 8.8</td>
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</table>

$P = 0.1$
were given 2 mg of this labeled material and then serially sacrificed. The activity of hematin extracted from the kidneys (after correction for total recovery) was related to the hematin activity of the test dose. 1 hr after the injection of labeled hemoglobin, when about 40% of the iron was taken up in the kidney, only one-third of this (or 15%) was present in intact hematin (Fig. 2). By 3 hr postinjection the hematin activity had fallen to 1%, although the total iron activity had not changed at all. There was a further decrease in

hematin activity over the next 12 hr. A parallel experiment in which $^{59}$Fe hematin was extracted gave comparable results (Fig. 2).

_Fate of Globin._—Hemoglobin labeled only in its protein with $^{14}$C-leucine was given to haptoglobin depleted rats. As shown in Fig. 3 there was a progressive decrease in the activity of TCA-precipitable kidney protein recovered during the first 16 hr. Renal uptake and retention of $^{59}$Fe activity in a parallel experiment is shown for comparison.

In 16 hr the total cumulative urinary $^{14}$C activity was 2.6 ± 1.0%, none of which was in protein.
DISCUSSION

The renal handling of hemoglobin has been studied by a variety of experimental approaches. Hemoglobin has been given in large excess to animals as a model for the acute renal failure which sometimes follows massive hemolysis. Others have used hemoglobin as a marker in studies of tubular absorption of protein. Stop-flow data (4, 5) have indicated that the proximal tubule is primarily involved. This has been well confirmed by morphologic studies following the administration of hemoglobin to animals. Unfortunately, in most of these, excessive doses were necessary to bring out the desired histologic detail. It is impossible to determine to what extent the changes observed were due to a toxic effect of hemoglobin. In some studies (3) heterologous hemoglobin was used. In others, intraperitoneal or intramuscular routes of injection were employed, rendering it difficult to follow the time course of renal uptake and subsequent degradation. In the experiments reported here, radioactive labeling of both heme and globin has provided a very effective way of following the fate of a small amount of intravenously injected hemoglobin. Animals were depleted of haptoglobin by injecting unlabeled hemoglobin prior to the radio-

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**FIG. 3.** Kidney globin activity. Haptoglobin-depleted rats were given 2.0 mg of oxyhemoglobin labeled only in the globin with $^{14}$C-leucine (○), and then serially sacrificed over the next 16 hr. The TCA-precipitable protein activity in the kidneys, corrected for incomplete recovery, is expressed as a per cent of the $^{14}$C activity injected. In a parallel group, receiving $^{59}$Fe oxyhemoglobin (●), the total kidney activity is expressed as a per cent of injected activity as in Fig. 1.
active test dose. In this way, a predictable amount of labeled hemoglobin (37 ± 3%) was taken up by the kidneys. No appreciable hemoglobin could be detected in the urine. Kidney uptake, then, was presumed to involve absorption of the filtered hemoglobin by the proximal tubule.

Rats given 65Fe hemoglobin showed a prolonged retention of labeled material in the kidneys. By 37 days, 54% of the original kidney activity still remained. Whatever kidney activity was lost was probably mobilized into the general body iron pool, as indicated by the following observations. The urinary excretion of 65Fe amounted to only 1.5% in 25 days. Furthermore, total body activity 25 days postinjection was no lower than in the rats that received a comparable amount of 65Fe hemoglobin but had no renal uptake because of the presence of haptoglobin. Finally, as shown in Table I, there was at least as much activity accounted for in the red cells and organs of the haptoglobin-depleted groups as in the control group 37 days after injection. Thus, whatever hemoglobin iron was taken up by the rat kidney appeared to be reutilized at a very slow rate.

Sears et al. (8) showed a similar fate for hemoglobin injected into a normal human volunteer. After a 19 g intravenous infusion, 1.8 g were excreted as urinary hemoglobin. The presence of hemoglobinuria implies that maximal tubular absorption of hemoglobin had occurred. Nevertheless, only 0.5 mg of nonhemoglobin urinary iron was excreted in 48 hr, an amount equivalent to 150 mg of hemoglobin. During the period from 12-48 hr after the infusion, the nonhemoglobin urinary iron excretion was 12 μg/hr, a value only somewhat higher than the basal (preinfusion) urinary iron excretion of 8 μg/hr. These data imply that the absorbed kidney hemoglobin iron was either excreted extremely slowly or was mobilized into the body iron pool. A number of postmortem examinations of patients with paroxysmal nocturnal hemoglobinuria (PNH) have shown marked iron deposition in the kidneys, mainly as hemosiderin in the proximal tubular cells, accompanied by depletion of total body iron stores. Thus the kidney deposits may not supply iron rapidly enough to prevent the patient from becoming iron deficient. In an electron micrographic study of the kidney in PNH, Reger and associates showed iron micelles indistinguishable from ferritin in the peritubular capillary endothelium and lumen (9). This iron may have been on its way toward reutilization.

Although the kidney iron activity fell only slowly after injection of the test dose, the iron appeared to be rapidly removed from its surrounding porphyrin. 1 hr after injection of 14C heme-labeled hemoglobin, only 15% could be recovered as kidney hematin. Thus, about two-thirds of the iron was then present in some other form. By 3 hr only 2% of the kidney iron was recoverable as hematin. The kidney must rapidly catabolize the heme moiety. Ostrow, Jandl, and Schmid (10) found that even though half of the 65Fe activity of doubly labeled rat hemoglobin was recovered in the kidneys, there was nearly
complete recovery of \(^{14}C\) activity as bilirubin within 6 hr after injection. Miller (3) found that ferritin did not appear in the normal tubules until 15 hr after the injection of large amounts of hemoglobin into mice. Perhaps the cells' capacity to degrade hematin can be overwhelmed by large doses. There may be a lag between the cleavage of the porphyrin ring and the transformation of tubular iron into ferritin. Apoferritin may have to be mobilized or synthesized in situ before the iron can be incorporated.

Experiments in which hemoglobin was labeled only in the protein with the precursor \(^{14}C\)-leucine showed the bulk of the absorbed hemoglobin was broken down within the 1st hr. These results indicate that absorbed globin is either rapidly transported out of the kidney intact or is catabolized in situ. The former seems unlikely since globin, free of heme, is extremely unstable. Even under ideal laboratory conditions it denatures and precipitates quite readily. It is difficult to imagine such a fragile protein traversing the tubular cell unscathed.

The renal handling of protein has been extensively studied in the rat. Sellers et al. have shown that normal rats excrete relatively more urinary protein than man (11). By the use of Evans Blue (T-1842) marker, they estimated that rat glomerular filtrate contained 0.08 mg/ml protein.\(^3\) The rate of tubular absorption was calculated to be 5 mg/hr, while the rate of urinary excretion was only 0.5 mg/hr. This absorbed protein may be metabolized in situ. When large amounts of either isologous or heterologous protein are given to rats, the filtered protein increases and marked proteinuria ensues. The proximal tubular cells become laden with vacuoles for a variable period of time following protein injection. Rather postulated that the tubular cell engulfed protein by athrocytosis, forming droplets which were then digested in situ (13). Recently Ericsson (2, 14) has presented ultrastructural studies of rat kidney after the administration of large (700 mg) doses of hemoglobin. His findings suggest that absorbed hemoglobin protein is actively catabolized by the proximal tubular cells.

The kidney may be important in the breakdown of proteins small enough to be filtered. Recent studies by Wochner, Strober, and Waldmann indicate that in the mouse, the kidney was the major site for the catabolism of light chains isolated from immunoglobulins and of Bence Jones proteins (15). Perhaps other low molecular weight proteins, such as polypeptide hormones, are also partly degraded in like manner.

A diagram outlining the proposed mechanism for the renal catabolism of hemoglobin is shown in Fig. 4. Hemoglobin is filtered through the glomerulus primarily as \(\alpha\beta\)-dimers (1) and then absorbed by the proximal tubular cells.

\(^3\) Using micropuncture technique, Van Liew et al found that the protein concentration of glomerular filtrate in the rat was about 0.15 mg/ml (12).
The hemoglobin iron detaches rapidly from the surrounding porphyrin and is stored in the cell as ferritin. The globin is probably catabolized in situ, perhaps under the influence of cytoplasmic lysosomes. When a large amount of hemoglobin is presented to the proximal tubule, absorptive capacity is exceeded and free hemoglobin appears in the urine. Furthermore, under these conditions absorbed iron is released into the tubular fluid and ultimately into the urine.

![Diagram](image)

**Fig. 4.** Proposed mechanism for the handling of hemoglobin by the rat nephron.

**SUMMARY**

The fate of small doses of isotopically labeled isologous hemoglobin was studied in the rat. When haptoglobin depleted animals were given 2.0 mg of \(^{59}\)Fe hemoglobin intravenously, nearly half was trapped by the kidneys. Kidney \(^{59}\)Fe activity disappeared slowly over several weeks. Whatever iron was lost from the kidneys was largely reutilized. In contrast, the porphyrin of hemoglobin absorbed by the kidneys appeared to be rapidly catabolized, since 5 hr after the injection of \(^{14}\)C or \(^{59}\)Fe heme-labeled hemoglobin only a small fraction was recovered as hematin. Likewise, after injection of globin-labeled hemoglobin, rapid disappearance of kidney protein activity indicated that the absorbed globin was readily catabolized in situ.

The technical assistance of Miss Nancyé Files is greatly appreciated.
BIBLIOGRAPHY


