Binding of acellular, native and cross-linked human hemoglobins to haptoglobin: enhanced distribution and clearance in the rat


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Ship, Noam J., Ayça Toprak, Roseanna P. Lai, Eric Tseng, Ronald Kluger, and K. Sandy Pang. Binding of acellular, native and cross-linked human hemoglobins to haptoglobin: enhanced distribution and clearance in the rat. Am J Physiol Gastrointest Liver Physiol 288: G1301–G1309, 2005; doi:10.1152/ajpgi.00399.2004.—It is well established that hemoglobin resulting from red cell lysis binds to haptoglobin in plasma to form a complex. The increased molecular size precludes its filtration by the kidneys, redirecting it toward hepatocellular entry. Chemically cross-linked hemoglobins are designed to be resistant to renal excretion, even in the absence of haptoglobin. The manner in which binding to haptoglobin influences the pharmacokinetics of acellular cross-linked and native hemoglobins was investigated after intravenous injection of radiolabeled native human hemoglobin and trimesyl-(Lys82)β-(Lys82)β cross-linked human hemoglobin, at trace doses, into rats. Under these conditions, there is sufficient plasma haptoglobin for binding with hemoglobin. In vitro binding assays by size-exclusion chromatography for bound and free hemoglobin revealed that, at <8 μM hemoglobin, native human hemoglobin was completely bound to rat haptoglobin, whereas only ~30% of trimesyl-(Lys82)β-(Lys82)β cross-linked hemoglobin was bound. Plasma disappearance of low doses (0.31 μM/kg) of native and cross-linked hemoglobins was monoeponential (half-life = 23 and 33 min, respectively). The volume of distribution (40 vs. 19 ml/kg) and plasma clearance (1.22 vs. 0.4 ml/min · 1−1 · kg−1) were higher for native than for cross-linked hemoglobin. Native and cross-linked human hemoglobins were found primarily in the liver, and not in the kidney, heart, lung, or spleen, mostly as degradation products. These pharmacokinetic findings suggest that the binding of hemoglobin to haptoglobin enhances its hepatocellular entry, clearance, and distribution.


THESE SPECIES ARE TERMED HEMOGLOBIN-BASED OXYGEN CARRIERS. THE CROSS-LINKED HEMOLOBINS DO NOT DISSOCIATE AND ARE NOT PRONE TO RENAL FILTRATION (5, 18, 20). BENESCH ET AL. (1) FOUND THAT HUMAN HEMOGLOBIN, WHEN CROSS-LINKED BETWEEN THE β-CHAINs WITH 2-NOR-2-FORMYLPIRIDOXAL, BINDS TIGHTLY TO HUMAN HAPTOGLOBIN IN VITRO, PRODUCING COMPLEXES THAT WERE READILY OBSERVED BY ELECTRON MICROSCOPY (44). HOWEVER, HIGH CONCENTRATIONS OF ADDED HEMOGLOBIN WOULD READILY EXCEED THE BINDING CAPACITY OF HAPTOGLOBIN IN PLASMA. AT HIGH DYES OF TETRAMERIC CROSS-LINKED HEMOGLOBINS [SPECIFICALLY, BIS(3,5-DIBROMOSALICYL)UMARATE AND 2-NOR-2-FORMYLPIRIDOXAL 5'-PHOSPHATE], ORGAN DISTRIBUTION (17, 19) AND CIRCULATION TIME (12) BECOME DOSE DEPENDENT.

WE HAVE UNDERTAKEN A THOROUGH COMPARISON OF NATIVE HUMAN HEMOGLOBIN AND A β-CHAIN [TRIMESYL-(LYS82)β-(LYS82)β] CROSS-LINKED HUMAN HEMOGLOBIN (24) TO UNDERSTAND THE PHARMACOKINETIC PROPERTIES OF SYNTHETIC, ACCELLULAR HEMOGLOBINS IN RATS IN VIVO. BECAUSE IT IS RECOGNIZED THAT THE FORMATION OF A COMPLEX BETWEEN HEMOGLOBIN AND HAPTOGLOBIN RESULTS IN LIVER CELL ENTRY AND CLEARANCE, BINDING OF THE CROSS-LINKED HUMAN HEMOGLOBIN TO RAT HAPTOGLOBIN SHOULD ALSO RESULT IN HEPATOCELLULAR ENTRY AND DEGRADATION IN RATS. IN THIS COMPARATIVE STUDY, WE TESTED THE HYPOTHESIS THAT THE CLEARANCE AND DISTRIBUTION OF HEMOGLOBIN ARE DIRECTLY CORRELATED TO ITS BINDING TO HAPTOGLOBIN UNDER CONDITIONS WHEREBY HAPTOGLOBIN IS IN EXCESS. WE ASSESSED THE BINDING OF NATIVE AND CROSS-LINKED HUMAN HEMOGLOBIN TO RAT HEMOGLOBIN IN RAT PLASMA AND EXAMINED THE INTRAVENOUS PHARMACOKINETICS OF LOW DOSES OF NATIVE AND CROSS-LINKED HUMAN HEMOGLOBIN. UNDER THIS CONDITION, THE DIFFERENTIAL HEMOGLOBIN BINDING TO HAPTOGLOBIN IS EXPECTED TO INFLUENCE THE IN VIVO DISPOSITION OF THE HEMOGLOBINS.
MATERIALS AND METHODS

Materials

Radiolabeled (3H and 14C) acetic anhydrides (50 and 5 mCi/mmol, respectively) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Distilled water was deionized with use of a Millipore filter. Protease inhibitor cocktail and a kit for the colorimetric assay for alanine aminotransaminase (ALT) were purchased from Sigma (St. Louis, MO).

Preparation of Human Hemoglobin

Human blood, collected by venipuncture, was washed three times with physiological saline solution (1:1 vol/vol) and centrifuged (15 min, 6,000 rpm) to provide red blood cells and plasma. The red blood cells were then lysed with two volumes of distilled and deionized water. The resulting cell fragments were pelleted by centrifugation (90 min, 20,000 rpm), and the supernatant containing hemoglobin was filtered (Millipore, 0.22 μm) to remove any residual cell debris. The hemoglobin solution was exposed to carbon monoxide (Boc Gas, Etobicoke, ON, Canada) and stored at 4°C. The concentration of hemoglobin was determined by measuring the absorbance of cyanmethemoglobin, prepared by reaction with potassium ferricyanide (41). Before the various treatments (cross-linking, acetylation, or use for binding studies), hemoglobin was separated from small-molecular-weight species in the hemolysate by size-exclusion chromatography (see below). The chromatographic steps, although successful in eliminating the small-molecular-weight species, failed to remove >25-kDa proteins. Examples of the proteins include catalase, superoxide dismutase, methemoglobin reductase, and glutathione peroxidase and transferase (14, 47). These proteins were present in fixed concentration ratios to hemoglobin among the samples and represented a small fraction of the total protein.

Human Hemoglobin Cross-Linked With Trimesoyl Tris(3,5-Dibromosalicylate)

Hemoglobin (2 ml, 4 mM), liganded to carbon monoxide, was exposed to bright-white light and bubbled with oxygen (Boc Gas) for 2 h at 0°C to exchange the carbon monoxide with oxygen. The solution was subjected to gel chromatography (Sephadex G-25 column, 5 × 25 cm, equilibrated with 0.1 M MOPS buffer, pH 8.0). Hemoglobin was then deoxygenated with nitrogen at 37°C for 2 h before the deoxygenated hemoglobin was cross-linked at the two β-Lys82 residues by reaction with a twofold excess of trimesoyl tris(3,5-Dibromosalicylate) (16 μmol, 16 mg) for 16 h at 37°C (24). The mixture was bubbled with oxygen to stop the cross-linking reaction, and the tetrameremic hemoglobin was separated from unreacted trimesoyl tris(3,5-Dibromosalicylate) by size-exclusion chromatography using a Sephadex G-25 column (2.5 × 25 cm) equilibrated with 0.1 M phosphate buffer, pH 7.4. The cross-linked hemoglobin was stored at 4°C under carbon monoxide. The purity of the cross-linked hemoglobin was found to be >99% cross-linked between the β-chains by C4 reverse-phase HPLC according to previously described methodology (24). The absence of monomeric β-chains was demonstrated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using an Applied Systems Voyager DE STR equipped with a 337-nm nitrogen laser (results not shown).

Hemoglobins Radiolabeled by Acetylation

The globin chains of hemoglobin or cross-linked hemoglobin (1 mM) were reacted with 3H- or 14C-labeled acetic anhydride for 20 min at pH 7 in 0.05 M acetic buffer. The mixture of radiolabeled species was resolved by size-exclusion chromatography (Sephadex G-25 column, equilibrated with 0.1 M phosphate buffer, pH 7.4). The eluted fractions corresponding to the product, i.e., labeled hemoglobin, were collected and pooled. The radiochemical purities of the 3H-labeled native and 14C-labeled cross-linked human hemoglobins, determined by ultracentrifugation through a 10,000-molecular-weight-cutoff membrane, were found to be >99.5%. The acetylated hemoglobins, monitored at 420 nm, displayed a retention time identical to that monitored by scintillation spectrometry, when the sample was subjected to chromatographic separation by a Superdex G-75 size-exclusion column, eluting with 0.1 M phosphate buffer, pH 7.4, at 0.4 ml/min. Acetylation of each of the hemoglobins was further confirmed by mass determination by means of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (results not shown). Before use, the sample, which had been stored as hemoglobin-carbon monoxide, was photoradiated under nitrogen, oxygenated, and then refrozen as hemoglobin-oxygen. These samples were thawed upon use for all binding and intravenous dosing.

Analysis of Human Hemoglobin Binding to Rat Haptoglobin

Rat plasma was taken from centrifuged blood that had been removed from the vena cava or by cardiac puncture. Rat hemoglobin was purified from the red blood cells as described above for human red blood cells. All hemoglobin solutions (native human and cross-linked and native rat hemoglobin) were first purified by size-exclusion chromatography (Sephadex G-25 column, equilibrated with 0.05 M Tris, pH 7.0).

Native human hemoglobin, cross-linked human hemoglobin, or rat hemoglobin was added to a fixed amount of rat plasma (10 μl) containing endogenous rat haptoglobin (~1 ng/ml, as determined by rat hemoglobin titration (4)). The mixtures were diluted with Tris buffer to a total volume of 20 μl and left at room temperature for 30 min for native (human or rat) hemoglobin and for 30 min, 4 h, and 24 h for cross-linked hemoglobin. For direct demonstration of binding, mixtures of rat plasma and hemoglobin (40–120 μg in 2 μl of plasma) or cross-linked hemoglobin (30–80 μg in 2 μl of plasma) were loaded onto the origin and allowed to run down a 6% polyacrylamide Tris-glycine gel. At 90 min, the gel was bathed in a mixture of 0.1% benzidine hydrochloride solution (10 ml) and hydrogen peroxide (40 μl) for 20 min to stain specifically for hemoglobin (3).

In a separate study, the entire 20-μl mixture was injected into an S-200 Sephacryl size-exclusion column, equilibrated with 0.5 M MgCl2 and 25 mM Tris buffer, pH 7.4, and detected by a UV/Vis detector at 420 nm. Hemoglobin and hemoglobin bound to haptoglobin absorb strongly at 420 nm, whereas haptoglobin and the other components of rat plasma do not. The identity of each hemoglobin was determined by comparing the retention times with those of human hemoglobin and cross-linked hemoglobin on their own and that of rat hemoglobin bound to rat haptoglobin in rat plasma (4, 39).

Resolution of Hemoglobin From Degradation Products in Biological Samples

The plasma sample, liver supernatant, and urine samples collected at 30 and 75 min were analyzed by size-exclusion chromatography. A Superdex G-75 column (4 × 30 cm) with a Shimadzu pump system (0.1 M phosphate buffer, pH 7.4, 0.4 ml/min), together with a Shimadzu detector (420 nm) and a sample collector (90 s), was employed to determine the fraction of the total radioactivity associated with radioactive hemoglobin and hemoglobin-haptoglobin (4, 39). Characterization of the biological samples was further enhanced by radioelution. The radioactive eluent, collected at 1.5-min intervals, was counted on a beta counter (model 5801, Beckman Coulter, Mississauga, ON, Canada) after the addition of 5 ml of Ready Protein scintillation cocktail. Radiolabeled hemoglobin bound to haptoglobin and hemoglobin (retention times between 20 and 30 min, respectively) absorbed strongly at 420 nm, whereas the degradation metabolites (determined from HPLC radioelution, retention times between 40 and 60 min) did not absorb (Fig. 1).
Rat Studies

Male Sprague-Dawley rats (314 ± 21 g) were obtained from Charles River (St. Constant, QC, Canada). Rats were given water and food ad libitum and kept under a 12:12-h dark-light cycle. The protocols were approved by the University of Toronto Animal Care Committee. The rats were anesthetized with pentobarbital sodium (50 mg/kg ip). Two sets of experiments, both involving intravenous dose administration, were performed.

Pharmacokinetic studies. In the first set of studies, a midline incision was made at the neck region. Then rats were cannulated at the jugular vein and carotid artery with polyethylene (PE-20 tubing) cannulas that were prefilled with heparinized physiological saline solution (200 U/ml) for dose introduction and blood sampling, respectively (36). A single dose of native human hemoglobin (22 ± 7 mg/kg or 0.344 ± 0.011 μmol/kg, 14C-radioactive dose = 9.2 ± 0.5 × 10^5 dpm) or cross-linked human hemoglobin (22 ± 1 mg/kg or 0.344 ± 0.016 μmol/kg, 3H-radioactive dose = 2.3 ± 0.7 × 10^6 dpm), in phosphate-buffered saline, was injected via a three-way stopcock into the jugular vein followed by diluted heparinized saline. The injected dose was ascertained by the difference in weight of the syringe-filled dose (in milliliters, assuming a density of 1 g/ml), before and after injection, multiplied by the concentration of the dose solution. The dose used for injection was further corrected for the radioactivity trapped in the injection device, ascertained in the wash fluid pursuant to dose injection. Blood was serially removed (0.2 ml) via a three-way stopcock from the artery at 5, 10, 15, 20, 30, 45, 60, 90, and 120 min. The blood samples were centrifuged (4 min, 6,000 rpm) to sediment the red blood cells to provide plasma. Some rats that received intravenous injections of radiolabeled hemoglobin were kept for 24–72 h for urine collection. For these rats, the cannulas were removed under anesthesia on completion of blood sampling (2 h), and the midline incision was resutured. Rats were kept in a Nalgene metabolism cage and given food and water until they were killed. Plasma was obtained immediately before the rats were killed by cardiac puncture under anesthesia. The plasma was subjected to a colorimetric assay for the enzyme ALT.

Organ distribution studies. In the second set of studies, organs and tissues were harvested after intravenous injection. Cannulation of the jugular vein and a V-line abdominal incision for bile duct cannulation (PE-20 tubing) were performed under anesthesia. A single dose of human hemoglobin (17 ± 7 mg/kg, 14C-radioactive dose = 5 ± 2 × 10^5 dpm) or cross-linked hemoglobin (1.8 ± 0.7 mg/kg, 3H-radioactive dose = 8 ± 3 × 10^6 dpm) in phosphate-buffered saline was injected into the vein. At 5, 15, 30, 45, 60, 90, or 120 min, the portal vein was cannulated (14-in. double-gauge cannula) and flushed with 20 ml of ice-cold physiological saline solution; the lower abdominal vena cava was immediately severed to allow the washing fluid to drain. The bladder was emptied of urine through suction by syringe. The liver, heart, lungs, spleen, and kidneys were removed, weighed immediately, and kept at −80°C until analyses. Upon thawing in ice, the organs (kidney, liver, heart, spleen, and lungs) were added to three parts of saline solution and homogenized over ice. The protease inhibitors before homogenization. After centrifugation (10 min, 12,000 rpm), 500 μl of the supernatant were added to 5 ml of Ready Protein (Beckman) and subjected to liquid scintillation spectrometry (model 5801, Beckman). The efficiency of counting in each homogenized organ was determined by calibration curves prepared by the addition of known disintegrations per minute of radiolabeled hemoglobin to blank tissues prepared in an identical manner.

Data Analyses

The concentrations (dpm/ml) of intact native and cross-linked human hemoglobin were normalized to the respective doses to yield fractions of dose per milliliter. The data were best fit to a first-order decay (the optimized fit) by nonlinear regression with the appropriate weighting procedures (Scientist, Micromath). The initial concentration (C0) was given by the y-intercept at time 0, and the volume of distribution was estimated by dose/C0. The area under the curve (AUC) for this first-order process was given by C0/k, where k is the first-order decay constant that bears a reciprocal relation to the half-life (k = 0.693/t1/2, where t1/2 is half-life); clearance was estimated as dose/AUC.

RESULTS

Binding of Native and Cross-Linked Human Hemoglobins to Rat Haptoglobin

The binding of human hemoglobin to rat haptoglobin in rat plasma was probed in a native gel (Fig. 2) and chromatographically (Fig. 3). The native gel showed two bands for human hemoglobin were normalized to the respective doses to yield
hemoglobin (lanes 4–7) and cross-linked human hemoglobin in rat plasma (lanes 8–11) when stained with benzidine and hydrogen peroxide. A limitation of this staining method is that the intensity in color has a greater dependence on the peroxidase activity of the protein than on the actual concentration of hemoglobin. Although the method reveals binding of the hemoglobin species to haptoglobin, it has long been known that the peroxidase activity of hemoglobin is vastly increased on binding to haptoglobin (1), and the method is only semiquantitative for assessing the degree of binding. Furthermore, Bunn (4) showed that the hemoglobin-haptoglobin complex may not be stable when it is subjected to electrophoresis, showing that the observed complex underestimates the true binding.

Mixtures of bound and free hemoglobin in rat plasma were characterized by size-exclusion chromatography, which provided a more quantitative view of the degree of complexation or hemoglobin-haptoglobin binding. The method is based on the differential retention times (RT) of native hemoglobin and cross-linked hemoglobin (64 kDa, RT = 40 and 37 min, respectively) and their haptoglobin-bound complexes (>140 kDa, RT = 30 and 20–27 min, respectively) and the strong absorbance of these hemoglobin species at 420 nm compared with other species in blank plasma at 420 nm (Fig. 3). Rat hemoglobin was mixed with rat plasma and used as the control sample for determining the retention time of lysis-derived rat hemoglobin that was present in rat plasma samples and bound to haptoglobin (RT = 30 min). Total hemoglobin was taken as the sum of the haptoglobin-bound and free hemoglobin for each plasma measurement, and this changed with added hemoglobin, native and cross-linked, suggesting that the bound and free hemoglobins exhibit the same absorbance. When the amount of human hemoglobin was increased, the area of the first peak, representing the hemoglobin-haptoglobin complex, increased proportionally to a certain point and then did not increase further. A later peak representing free hemoglobin emerged with the addition of more hemoglobin (Fig. 3A), suggesting that haptoglobin’s binding sites were saturated. Conversely, the first and last peaks increased in concert with addition of cross-linked hemoglobin to rat plasma (Fig. 3B), suggesting that the unbound fraction remained rather constant with dose. When data were presented as the haptoglobin-bound fraction, it was readily seen that the binding of native human hemoglobin and rat hemoglobin to the same amount of haptoglobin in rat plasma was almost complete and tighter than that of cross-linked human hemoglobin (Fig. 4). At ≥8 μM human or rat hemoglobin, haptoglobin became saturated, and any excess hemoglobin resulted in a decrease in the fraction of bound hemoglobin. In contrast, the binding of cross-linked hemoglobin to rat haptoglobin was lower (30% bound) and relatively constant (Fig. 4).

Fig. 2. Native gel of human hemoglobin bound to rat haptoglobin in plasma. The 6% Tris-polyacrylamide gel was stained with 0.1% benzidine and hydrogen peroxide for 20 min. Lane 1, human hemoglobin; lane 2, cross-linked human hemoglobin; lane 3, rat plasma (2 μl in each of the following lanes); lanes 4–7, increasing amounts of hemoglobin in rat plasma (40–120 μg); lanes 8–11, increasing amounts of cross-linked hemoglobin in rat plasma (30–80 μg).

Fig. 3. Size-exclusion chromatography of human hemoglobin (A) and cross-linked human hemoglobin (B) in rat plasma containing endogenous rat haptoglobin (Hp). Separation was performed on a Sephacryl S-200 column at the flow rate of 0.4 ml/min with a mobile phase of 0.5 M MgCl₂ in 25 mM Tris buffer, pH 7.4. Different amounts of hemoglobin (3.9, 5.2, 7.7, 10.3, and 15.5 μg; A) or cross-linked hemoglobin (2.6, 5.2, or 7.8 μg; B) were added to 10 μl of rat plasma and left to equilibrate for 30 min. Arrows 1 and 2 in A, elution times for haptoglobin-bound hemoglobin and free hemoglobin, respectively. Arrows 1, 2, and 3 in B, elution times for haptoglobin bound to cross-linked hemoglobin, rat haptoglobin bound to endogenous rat hemoglobin, and free cross-linked hemoglobin, respectively.
Disappearance of Native and Cross-Linked Human Hemoglobins From the Rat Circulation

During the 1st h after intravenous injection, most of the total radioactivity in the plasma represented intact hemoglobin, as assayed by size-exclusion chromatography (Fig. 5). However, more degradation products appeared with time. The concentration of intact native and cross-linked hemoglobin, over time, fit best to a first-order process (Table 1). The volume of distribution and clearance were statistically greater for native than for cross-linked hemoglobin (Fig. 6, Table 1). The half-life of native hemoglobin was also shorter (23 min) than that of cross-linked hemoglobin (33 min).

Biodistribution of the Hemoglobins

The greatest accumulation of radioactivity occurred at 60 min in the liver (Fig. 7, Table 2). The injected radiolabel was initially associated with intact radiolabeled native or cross-linked hemoglobin in the plasma (Fig. 1A), but among the liver samples, the radioactive species that eluted later than intact hemoglobin did not display an absorbance at 420 nm (Fig. 1B). These findings imply that the radioactive species in liver are metabolites of hemoglobin. The other organs (kidney, spleen, heart, and lungs) retained only limited amounts of the dose over the entire experiment (Fig. 7, Table 2). No significant amount of radiolabel was recovered in the bile, and the urine accumulated <2% of the injected dose. However, for rats that were kept for >3 days, the excretion in urine and feces represented <15% of the total dose. For all the rats examined, there was no elevation of the enzyme ALT in the plasma at the time of death, normally an index of liver damage.

DISCUSSION

The binding of human hemoglobin to rat haptoglobin was deemed to be an efficient process on the basis of the observation that all the hemoglobin added to the rat plasma appeared as a haptoglobin-bound complex of higher molecular weight until full haptoglobin saturation was reached (Fig. 4). Native human and rat hemoglobin exhibit almost complete binding to rat haptoglobin. On the other hand, complex formation of cross-linked human hemoglobin with rat haptoglobin was less tight and incomplete, even at low concentrations, and mirrored the binding results of dimethyl adipimidate cross-linked hemoglobin (30). Bunn (4) reported similar findings in his study of human hemoglobin that had been cross-linked with bis-(N-maleimidomethyl)ether between the β-chains in human plasma and found that native hemoglobin was able to displace some of the cross-linked hemoglobin that was already bound.

![Image](http://ajpgi.physiology.org/)
In addition to differences in binding to haptoglobin, the pharmacokinetics for the native and the trimesyl-(Lys82)β-(Lys82)β cross-linked hemoglobin species also diverged. Although the mode of decay for each hemoglobin was first order, conforming to a single-compartment model, the extrapolated concentration at time 0 of unmodified hemoglobin was only half that of cross-linked hemoglobin (Fig. 5, Table 1). When this concentration was divided by the dose, the volume of distribution for unmodified native hemoglobin (40 ml/kg) was double that of cross-linked hemoglobin (19 ml/kg). The clearance of native hemoglobin, obtained as dose/AUC, was almost three times that of cross-linked hemoglobin. The difference in clearance for the two hemoglobins was not due to differences in renal excretion, because very low levels of radioactivity were recovered in urine (Table 2). As a result of the faster clearance, the half-life of native hemoglobin was shorter (23 min), despite the larger accessible volume. The half-life for cross-linked hemoglobin in the circulation was prolonged (33 min) because of the reduced clearance. It should be noted that the half-lives for the hemoglobins under transfusion loads would be altered when binding to haptoglobin and internalization mechanisms are saturated, and elimination by the liver is suboptimal (12, 20).

The lower clearance of cross-linked hemoglobin may be a consequence of incomplete binding to haptoglobin (Fig. 4), suggesting that the haptoglobin-bound complex enters the liver via specific receptors for hemoglobin–haptoglobin. This finding concurs with those of others (21–23, 32, 48). However, a faster entry of free hemoglobin had been suggested in isolated hepatocytes prepared by collagenase digestion (43). Whether this phenomenon holds for the intact liver is uncertain, because loss of receptors for internalization into hepatocytes prepared by collagenase has been reported (22, 48).

Our organ distribution study revealed that native and cross-linked hemoglobin species accumulated in the liver and most of the radioactivity appeared as degradation products, on the basis of the extended retention time of the radiolabeled species over hemoglobin (Table 2, Fig. 1B). Half of the total radioactivity injected into the rat circulation accumulated in the liver after 1 h. The accumulation profiles of total radioactivity of both hemoglobins in liver over the 2-h period were quite similar to one another (Fig. 7). The accumulation of total activity was also similar to that reported by Higa et al. (13) and Oshiro and Nakajima (34), who injected the bound complex of rat hemoglobin and rat haptoglobin into rats. The composition of hemoglobin metabolites in our work was, however, unknown, because the radiochromatograms showed only the products that retained the radiolabeled-acetylated species, and not all products were radiolabeled. It may be concluded that, for the radiolabeled products detected at retention times >40 min, the labeled metabolites were of smaller molecular size than hemoglobin (Fig. 1) and were a mixture of small molecules. Similarly, Higa et al. (13) and Oshiro and Nakajima (34) observed the gradual degradation of 125I-labeled globin chains of hemoglobin into labeled species of smaller molecular size within the liver. However, only a small amount of radioactivity appeared in bile. The observation suggests the high likelihood the metabolites formed within the liver must have reentered the circulation. The labeled degradation products that reentered the plasma could be subject to distribution, binding, and incorporation into tissue sites. Hence, use of total radioactivity in the estimation of clearances or volumes of distribution of the hemoglobins would lead to errors. Indeed, this was found in our preliminary sets of experiments in which we had not distinguished the hemoglobin from its degradation products (unpublished data). We detected changes in volume, but not in clearance, in a two-compartment fit for the native human and cross-linked hemoglobins (unpublished data). Yet the present interpretation that correctly utilized unchanged hemoglobin

Table 1. Pharmacokinetic parameters of native human (14C-acetylated) and cross-linked (3H-acetylated) human hemoglobin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Native hemoglobin</th>
<th>Cross-linked hemoglobin</th>
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<tbody>
<tr>
<td>Mean ± SD (n = 4)</td>
<td>324 ± 10</td>
<td>301 ± 21</td>
</tr>
<tr>
<td>Dose, mg/kg</td>
<td>22 ± 7</td>
<td>22 ± 1</td>
</tr>
<tr>
<td>Co, % dose/ml</td>
<td>8.2 ± 2.3</td>
<td>18.3 ± 5.0</td>
</tr>
<tr>
<td>Va, ml/kg</td>
<td>40 ± 13</td>
<td>19 ± 5</td>
</tr>
<tr>
<td>k, 1st-order decay constant</td>
<td>3.0 ± 0.4</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>t1/2, min</td>
<td>23 ± 3</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>AUC, (% dose/ml-min)</td>
<td>2.7 ± 0.6</td>
<td>8.0 ± 2.0</td>
</tr>
<tr>
<td>CL, ml·min⁻¹·kg⁻¹</td>
<td>1.22 ± 0.37</td>
<td>0.40 ± 0.05*</td>
</tr>
</tbody>
</table>

Pharmacokinetic parameters were determined by fitting data from each experiment to a 1st-order decay; error represents standard deviation of the mean. All data from the 4 experiments were pooled and fit to a 1-compartment model; error represents standard deviation of the fitted parameter. Co, initial concentration; Va, volume of distribution; k, 1st-order decay constant; t1/2; half-life; AUC, area under the curve; CL, clearance. *P = 0.011 vs. native hemoglobin; **P = 0.006 vs. native hemoglobin.

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Fig. 6. Fates of acellular, native [3H]hemoglobin (open symbols, n = 4) and cross-linked [14C]hemoglobin (solid symbols, n = 4) after their respective intravenous injections into jugular veins of rats. Concentrations of intact, human cross-linked hemoglobin (1 symbol for each study) revealed a slower 1st-order decay than did native hemoglobin (1 symbol for each study). Parameters, summarized in Table 1, were derived from fits to 1st-order decays (1-compartment model).
data pointed to a one-compartment model fit and differences in volume and clearance (Table 1).

The general belief is that the hemoglobin, cross-linked between the β-subunits, extends its circulatory retention time by preventing renal filtration. For cross-linked hemoglobin, it has been generally accepted that cross-linking hemoglobin increases its circulatory retention time as a result of improved stability and reduced renal filtration. Kidney filtration of native hemoglobin occurs only when the available haptoglobin is saturated, but excretion is not an important clearance pathway under the low-concentration conditions studied here. Our findings suggest that native and cross-linked hemoglobin are cleared mainly by the liver, and little radioactivity was recovered in the kidneys or the urine for hemoglobin or cross-linked hemoglobin. These findings infer that low doses of hemoglobins are not toxic to the kidneys, although the situation will be worsened when high levels of acellular, native or cross-linked hemoglobin is transfused. Haptoglobin is present only at 0.1 g/100 ml in blood, and transfusions of hemoglobin-based oxygen carriers range from 1 to 14 g of hemoglobin per 100 ml (12, 31, 35, 40). Under these other conditions, haptoglobin binding will be saturated. The transfused cross-linked hemoglobin will compete with exogenous native hemoglobin for haptoglobin binding, and the bound hemoglobin-haptoglobin and cross-linked hemoglobin-haptoglobin complexes compete for receptors for their internalization into liver parenchymal cells (43). If haptoglobin is depleted by the excess cross-linked hemoglobin, acellular native hemoglobin clearance will involve the kidneys more than the liver, as observed by Hershko et al. (11), and kidney damage will ensue (27, 28).

Table 2. Distribution of total radioactivities in organs and fluids 60 min after intravenous injections of labeled hemoglobins

<table>
<thead>
<tr>
<th></th>
<th>Body wt, g</th>
<th>Liver</th>
<th>Bile</th>
<th>Kidney</th>
<th>Urine</th>
<th>Spleen</th>
<th>Lung</th>
<th>Heart</th>
<th>Plasma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native hemoglobin</td>
<td>310±30</td>
<td>46±14</td>
<td>1.8±0.6</td>
<td>2±2</td>
<td>0.2±0.2</td>
<td>3±1</td>
<td>0.7±0.2</td>
<td>0.4±0.1</td>
<td>1.3±1.1</td>
<td>55±15</td>
</tr>
<tr>
<td>Cross-linked hemoglobin</td>
<td>320±20</td>
<td>45±15</td>
<td>0.5±0.3</td>
<td>3±2</td>
<td>0.5±0.2</td>
<td>3±3</td>
<td>1.0±1.0</td>
<td>0.5±0.5</td>
<td>2.6±0.3</td>
<td>56±15</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 3. Disintegration per minute in organ and plasma at 60 min were normalized to dose to provide amounts in tissue or organ (%dose). For bile and urine, cumulative amounts of radioactivity collected from time 0 to 60 min were normalized to dose.
The studies described here presented an anomaly that drug binding to plasma proteins increased clearance and biodistribution. This concept is contrary to most pharmacokinetic principles that forecast reduced clearance and diminished distribution with increased vascular protein binding (7, 26, 37, 45). The observation that protein binding promotes distribution and clearance is possible only in the case of receptor-mediated transport of the haptoglobin-bound species, and not the free hemoglobin. We found that reduced binding of trimesyl-(Lys82)β-(Lys82)β cross-linked hemoglobin with haptoglobin, compared with that of native hemoglobin, curtails the biodistribution volume. Future hemoglobin-based oxygen carriers should be designed to prevent complexation with haptoglobin-haptoglobin, compared with that of native hemoglobin, curtails the biodistribution volume. Future hemoglobin-based oxygen carriers should be designed to prevent complexation with haptoglobin, compared with that of native hemoglobin, curtails the biodistribution volume. Future hemoglobin-based oxygen carriers should be designed to prevent complexation with haptoglobin.

REFERENCES


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