Mechanisms of Plasma Hemoglobin Clearance after Acute Hemolysis in Dogs:

Serum Haptoglobin Levels and Selective Deposition in Liver and Kidney

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THE ACUTE HEMOLYSIS produced during extracorporeal circulation and the chronic hemolysis found after implantation of aortic or mitral ball-valve prostheses indicate a need for further information on the mechanisms involved in clearance of hemoglobin from circulating plasma.

The usual concept of removal of extracorpuscular hemoglobin from plasma is based on primary combination with circulating serum haptoglobin into a molecule which is not filtrable by the renal glomerulus and is removed from circulation by the reticulo-endothelial system; when all available haptoglobin has been combined with hemoglobin, further accumulation of *free* hemoglobin in the plasma results in hemoglobinuria.[7, 8, 11, 13]

If the kidneys function as effective excretory organs for hemoglobin, and if no other primary pathways for removal of free hemoglobin from the plasma exist, plasma hemoglobin in excess of that bound by haptoglobin should be excreted quantitatively in the urine. It was determined in a previous study of open-heart surgical patients,[2] however, that usually less than 10 per cent of the total amount of free, or unbound, hemoglobin circulating in plasma was excreted in urine, and it appeared that the remaining free hemoglobin was removed from plasma by other means, presumably by deposition in body tissues.

This report presents experimental studies in dogs to clarify mechanisms of clearance of plasma hemoglobin, with special reference to identification and quantification of the routes of removal of free hemoglobin not accounted for by urinary excretion or by combination with haptoglobin. Studies were also performed of subsequent rates of disappearance of hemoglobin from certain tissues in which it was found heavily deposited during the period of primary removal of hemoglobin from plasma.

Materials and Methods

Experiments were performed on 25 mongrel dogs weighing between 11 and 18 Kg. Animals were anesthetized lightly with intravenous sodium pentothal. An indwelling catheter was inserted into the bladder for continuous collection of urine. An intravenous catheter was inserted into the inferior vena cava for injection of hemoglobin and blood sampling. This catheter was also used for infusion of fluid (5% dextrose in water) during the 4- to 6-hour test period; the total amount of fluid given averaged

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TABLE 1. *Summary of Amount of Hemoglobin Injected, Maximum Plasma Hemoglobin Concentration Achieved, Maximum Serum Haptoglobin and Free Plasma Hemoglobin Levels, and Total Urinary Excretion of Hemoglobin**

Expt. No.	Hb. Inj. (Gm.)	Total Plasma Hb. (mg. %)	Serum Hpt. (mg. %)	Free Plasma Hb. (mg. %)	Total Urine Hb. (mg.)
6	0.305	122	126	0	0
25	0.660	152	142	10	0
26	0.785	174	160	14	0
24	0.327	175	155	20	0
23	1.896	304	243	61	0.072
9	2.031	327	265	62	0
3	0.608	325	248	84	0
22	1.570	186	101	85	0
17	1.354	209	125	85	0.022
14	2.968	329	241	88	3.920
21	1.635	300	208	92	0.03
15	1.950	226	116	95	0.13
13	2.028	299	157	96	0.46
5	0.327	199	94	105	0.57
2	0.951	265	142	123	12.30
18	1.698	257	125	132	0
7	0.667	215	95	135	0
4	1.044	213	143	144	0.64
12	2.028	298	128	162	0.27
19	2.094	264	102	164	0.03
20	1.662	336	150	186	0.35
16	2.688	400	134	266	2.78
8	2.232	374	85	289	117.3
10	1.959	418	92	326	0.95
1	1.900	470	140	331	34.2
11	1.612	434	82	338	2.92

* Arranged in ascending order of maximum *free* plasma hemoglobin levels in the various experiments.

approximately 500 ml., which maintained adequate urinary output in all experiments.

During a control period, blood samples were taken for determinations of serum haptoglobin and plasma hemoglobin and the urine was collected.

Hemoglobin solution, prepared as described below, was then infused intravenously in amounts estimated to result in plasma hemoglobin levels ranging from 50 to 400 mg.%. This hemoglobin was labeled with Cr^{51} to trace the sites of deposition of hemoglobin in body tissues.

After injection of hemoglobin the following were measured at hourly intervals for 4 hours: serum haptoglobin, plasma hemoglobin, urine hemoglobin concentration, and urine volume. Blood and urine samples were again collected the following morning.

Tissue distribution of Cr^{51} was determined by measuring the radio-activity level in biopsies taken at the time of autopsy or laparotomy on the day following hemoglobin injection.

In the last 13 animals studied, the dogs were kept alive for periods up to nearly 4 months and biopsies of liver and kidney were obtained at random intervals for measurement of tissue radio-activity. This part of the study was limited to measurements of liver and kidney since previous experiments indicated these organs to be the primary sites of hemoglobin accumulation. Biopsies were obtained by laparotomy performed under sterile conditions.

The hemoglobin solution was prepared as described by Gabrieli *et al.*[5] Fresh dog blood was collected and added to ACD and approximately 30 μc of Cr^{51} as $Na_2Cr^{51}O_4$ for each ml. of blood. The blood was centrifuged for 45 minutes, the supernatant removed, and the red cells washed with physiologic saline. Red cells were then lysed with distilled water. The hemolysate was centrifuged to remove particulate matter and then passed through a sephadex column for separation into the fraction in which Cr^{51} was virtually all hemoglobin-bound.[6] Samples of hemoglobin solution were then taken for measurement of radioactivity and hemoglobin concentration, and the dosage for administration calculated according to body weight of the dogs to produce the desired range of plasma hemoglobin concentration.

Plasma and urine hemoglobin determinations were obtained by a modified peroxidase method as described by Gabrieli and Pyzikiewicz.[4] Serum haptoglobin levels were measured by the method of Lionetti *et al.*[10]

Results

Hemoglobin and Haptoglobin Levels and Urinary Excretion. Graduated dosages of hemoglobin solution estimated to produce maximum plasma hemoglobin levels ranging up to 400 mg.% were administered to individual dogs. Table 1 lists the maximum plasma hemoglobin levels achieved and the serum haptoglobin level at the time of maximum plasma hemoglobin levels in each experiment. The maximum *free* plasma hemoglobin level was then calculated by subtracting the serum haptoglobin level from the maximum plasma hemoglobin level. Total output of hemoglobin in the urine was also tabulated, this figure representing the amount measured in the urine from the time of administration of hemoglobin until the urine became hemoglobin-free, usually within 4 hours.

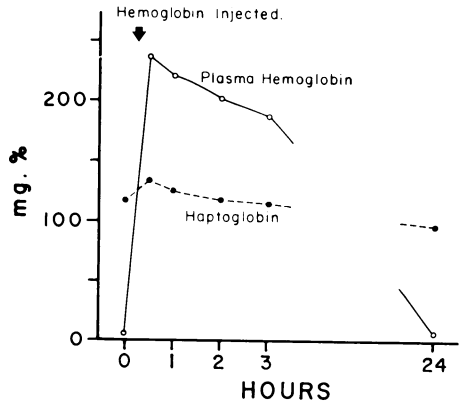

FIG. 1. Serial levels of total plasma hemoglobin and serum haptoglobin during the first few hours after hemoglobin injection and on the following morning.

Total plasma hemoglobin levels ranged from 122 mg.% to 470 mg.%. Maximum free plasma hemoglobin ranged from 0 to 338 mg.%.

Urinary excretion of hemoglobin in general did not bear a close quantitative relationship to levels of free plasma hemoglobin. However, of seven experiments in which the free plasma hemoglobin level was less than 85 mg.%, in only one did any hemoglobin appear in the urine, whereas hemoglobinuria occurred in all instances except two with free plasma hemoglobin levels of 85 mg.% or more.

It is evident that the total amount of hemoglobin excreted in the urine accounted for only a small percentage of the total pool of free plasma hemoglobin, suggesting that the kidneys did not function as effective excretory organs for hemoglobin and that the major portion of free hemoglobin was removed from the plasma by deposition in body tissues as documented below.

Haptoglobin and Hemoglobin Clearance Rates. Figure 1 illustrates serial haptoglobin levels during the first 4 hours after injection of hemoglobin and on the following morning. Haptoglobin levels decreased only slowly during the first 4 hours despite the fact that all serum haptoglobin

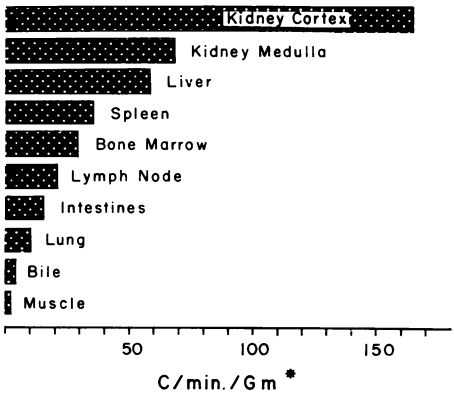

FIG. 2. Mean Chromium-51 concentration in various tissues on the dog following injection of labelled hemoglobin.* For purposes of data presentation, tissue radio-activity was converted to counts/min./Gm. tissue/10,000 counts/ml. of hemoglobin solution injected.

was combined with hemoglobin. Although plasma hemoglobin fell to near normal levels, on the following morning serum haptoglobin levels were only slightly below original levels, suggesting a rapid rate of regeneration of haptoglobin.

Deposition of Hemoglobin in Body Tissues. Figure 2 illustrates relative radioactivity levels in various body tissues, as measured at autopsy or laparotomy on the day following hemoglobin injection. The validity of the assumption that these radioactivity levels represent labelled hemoglobin has been discussed fully elsewhere,[4] and previous studies appear to justify this assumption. The heaviest concentration occurred in the kidney cortex with progressively lesser concentrations in kidney medulla, liver, spleen, bone marrow, lymph nodes, intestines and lungs. Negligible concentrations were also measured in bile and muscle.

The data in Figure 2 represent the first 12 experiments. In the subsequent 13 experiments only kidney and liver were studied since it was evident that these were the principle tissues involved.

Figure 3 illustrates the concentration of hemoglobin in liver and kidney cortex in relation to levels of free plasma hemoglobin in individual experiments. The concentra-

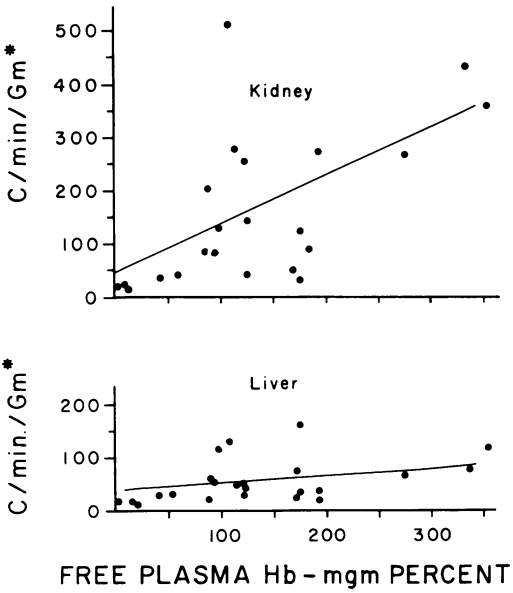

FIG. 3. The relationship of hepatic and renal tissue radioactivity levels (indicative of hemoglobin accumulation) to increasing free plasma hemoglobin concentrations.

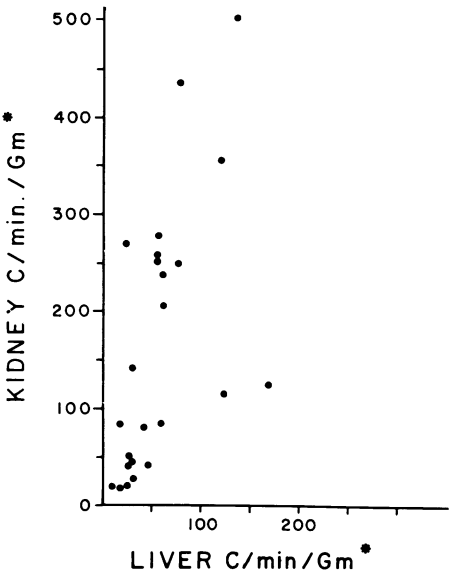

FIG. 4. Relative change in liver and kidney tissue radioactivity with increasing levels of plasma hemoglobin and tissue deposition.

tion of hemoglobin in the liver increased only slightly with increasing concentrations of free plasma hemoglobin, suggesting that almost all the deposited hemoglobin in the liver represented hemoglobin-haptoglobin complex. The concentration of hemoglobin in the kidney, on the other hand, showed a sharp and progressive increase with increasing concentrations of free plasma hemoglobin, suggesting that the renal cortex was the principal site of deposition of free plasma hemoglobin and that this deposition bore a direct relationship to the plasma hemoglobin concentration.

This relationship is further illustrated in Figure 4 in which respective concentrations of hemoglobin in liver and kidney are plotted for individual experiments. There was only a small tendency toward increasing concentration in the liver in instances with higher concentrations in the kidney, indicating again the selective deposition of hemoglobin in the kidney with increasing concentrations of free plasma hemoglobin.

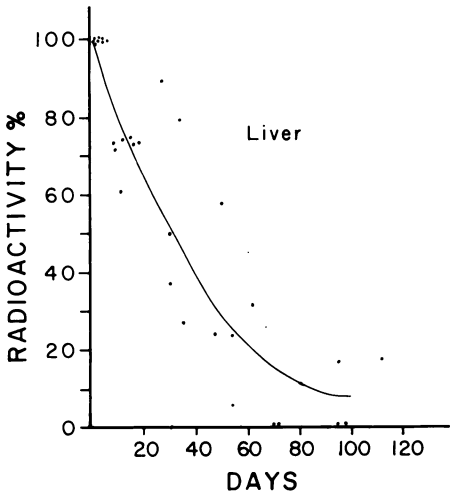

FIG. 5. The rate of decrease in tissue radioactivity in the liver during the 4-month period after the original injection of labelled hemoglobin. Radio-activity levels in biopsies taken the day following hemoglobin administration were taken as the "100%" level. Data were subjected to regression analysis on a digital computer to obtain the curve with best correlation.

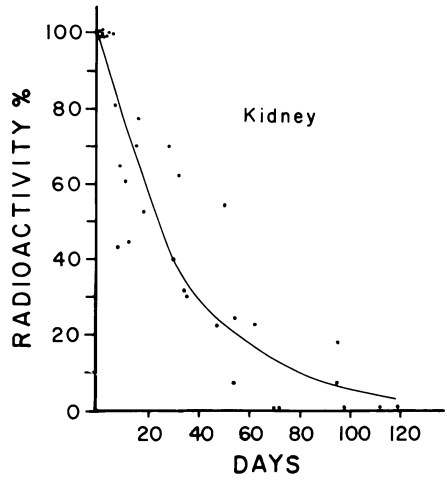

FIG. 6. The rate of decrease in tissue radioactivity in the kidney during the 4-month period after the original injection of labelled hemoglobin. Radio-activity levels in biopsies taken the day following hemoglobin administration were taken as the "100%" level. Data were subjected to regression analysis on a digital computer to obtain the curve with best correlation.

Remobilization of Hemoglobin from Liver and Kidney. Figures 4 and 5 illustrate the pattern of decreasing tissue radioactivity in liver and kidney during a 4-month period after hemoglobin injections. The concentration decreased in a nearly exponential fashion and fell to essentially zero in about 100 days. No appreciable difference was found between disappearance rates in liver and kidney. It was not possible to trace the subsequent fate of this remobilized hemoglobin, although no measurable hemoglobin was identified in the urine during early postinjection days by either urine radio-activity levels or direct urinary hemoglobin assay.

Discussion

It has long been known that under certain circumstances hemoglobin is excreted in the urine, and after demonstrating that renal excretion occurred only when plasma hemoglobin exceeded certain levels, Lichty *et al.*[9] developed a theory of a renal threshold for hemoglobin; a similar finding in hu-

mans was later observed by Ottenberg and Fox.[14] These observations remained unexplained until the demonstration by Laurell and Nyman[8] that a specific plasma protein, an alpha-2 globulin, haptoglobin, had the property of quantitatively binding free hemoglobin into a molecule which was too large to be filtered by the renal glomerulus. It was then confirmed by others[7, 11, 13] that the *renal threshold* of Lichty approximated serum levels of haptoglobin, and that it was only when the haptoglobin level in plasma was exceeded that hemoglobinuria occurred.

These observations necessitated a revision of the *renal threshold* concept as follows: free hemoglobin preferentially combines with available haptoglobin until the haptoglobin pool is saturated, and this complex is removed from circulation by the reticulo-endothelial system, chiefly in the liver; further accumulations of hemoglobin in the plasma then result in circulating free, or unbound, hemoglobin which is excreted by the kidneys.

Nevertheless, quantitative studies have been singularly lacking to substantiate the hypothesis that these two mechanisms would account entirely for the removal of hemoglobin from the plasma. There have been, in fact, a number of observations indicating that this is not a complete explanation and that some other mechanisms for removal of hemoglobin from the plasma exist.

Lathem's[7] studies of renal excretion of hemoglobin demonstrated that hemoglobin appeared in the urine only after the haptoglobin level was significantly exceeded rather than at the first appearance of free hemoglobin in the plasma; the measured amount of hemoglobin present in excess of the hepatoglobin level averaged 27 mg.% and ranged up to 60 mg.% before hemoglobinuria occurred. He concluded that evidence of tubular re-absorption was lacking and that the tubules did not play a significant role in regulating hemoglobin excretion. Lathem[7] also noted the problem of explaining permeability of the glomerulus to hemoglobin, which in molecular weight is similar to albumin, and raised the question as to whether the glomerulus is indeed permeable to free hemoglobin molecules or whether hemoglobin only enters glomerular filtrate after dissociation into smaller components. The latter possibility is unlikely since it has been shown that electrophoretic characteristics of urinary hemoglobin are in all respects similar to these of free plasma hemoglobin.[7]

Further evidence of other routes of hemoglobin clearance, in addition to combination with haptoglobin and renal excretion, were provided by clinical studies in patients undergoing open-heart operations[2] in whom it was shown that the percentage of available free hemoglobin which appeared in the urine was only a very small portion of that theoretically available for renal excretion.[2] Although it may be considered that renal function was not entirely normal in such postoperative patients, those observations are in agreement with the present experimental studies.

In previous studies of kinetics of plasma hemoglobin catabolism in rats, Gabrieli[4] noted that liver radio-activity after injection of Cr^{51} tagged hemoglobin increased during the first 2 hours, remained the same for a period of hours, then slowly decreased. He also found that the kidneys contained much Cr^{51}, suggesting deposition of hemoglobin in the kidneys. Murray[13] also demonstrated in rabbits that haptoglobin-bound hemoglobin accumulated primarily in the liver and that free hemoglobin accumulated in the kidneys, even though none appeared in the urine.

Certain variations in reported studies on plasma hemoglobin clearance appear to be related to species variations in experimental animals and this variation from man seems to apply also to dogs, which have an

apparently larger and more rapidly regenerated haptoglobin pool than humans. It has been demonstrated in humans that a single episode of severe hemolysis results in essentially complete disappearance of haptoglobin from the plasma; [1, 2] since haptoglobin is regenerated slowly it returns to normal only after a period of several days. However, Lichty's original experiments in dogs demonstrated the striking fact that, although a second injection of hemoglobin resulted in hemoglobinuria occurring at a lower plasma hemoglobin level than with the initial injection, the *renal threshold* then remained at essentially this slightly reduced level, and with repeated daily injections, high levels of plasma hemoglobin were required to produce hemoglobinuria. This phenomenon appeared to substantiate the concept of a *renal threshold* which was lowered by previous injection of hemoglobin but still continued to be present.

The present studies explain this aspect of Lichty's observations since it was noted that haptoglobin levels in dogs were only slightly depressed on the day following hemoglobin administration, in contrast to the essentially complete removal of haptoglobin from plasma previously observed in patients subjected to acute hemolysis during open-heart surgical procedures.[2] It appeared, therefore, that either large haptoglobin reserves are present in the dog and are mobilized upon demand or else the regenerative rate in the dog greatly exceeds that of man, accounting for relatively sustained haptoglobin levels during serial observations. These facts probably account for studies indicating that tolerance to injected hemoglobin is high and renal damage difficult to produce in the dog as compared to known effects in humans.

The present experiments suggest that renal deposition of hemoglobin is of importance not only during episodes of massive hemolysis but with hemolysis of any measurable degree in excess of the combining power of circulating haptoglobin. In patients whose serum is devoid of haptoglobin, as may occur in any chronic hemolytic state (*including patients with cardiac ball-valve prostheses*), even relatively low degrees of hemolysis result in the chronic production of free hemoglobin in the plasma. Free hemoglobin is then filtered out of the plasma and deposited in body tissues and particularly in the kidneys. With increasing levels of free hemoglobin selective deposition of hemoglobin in the kidneys becomes even more marked. Although this observation does not explain this accumulation in renal tissue, it does provide further information which may be related to renal failure in conditions associated with varying degrees of hemolysis.

The present findings also support previous observations [2, 7] suggesting that the kidneys do not perform as useful excretory organs for free hemoglobin. The seemingly random excretion of free hemoglobin in amounts which are small in relation to the amount in the plasma remains unexplained. However, the size of the hemoglobin molecule is critical in respect to pore size of the glomeruli, and this may account for the lack of a more predictable excretion. It may also be that the renal vasoconstriction with reduction in renal blood flow and glomerular filtration rate which accompanies intravenous injection of hemoglobin plays a role in this pattern.[12]

Studies of rate of remobilization of deposited hemoglobin from liver and kidney cannot be considered entirely conclusive since it is not possible to be certain that disappearance of Cr^{51} from the tissues actually represents disappearance of hemoglobin or only liberation of chromium from the hemoglobin molecule. Nevertheless, it does indicate persistence of hemoglobin in the tissue for significant periods of time and therefore suggests the likelihood of progressive accumulation in chronic hemolytic states in which the rate of daily hemolysis

(and tissue deposition) may exceed the rate of remobilization of hemoglobin from the tissues.

Although one must be cautious in applying observations from experimental animal to responses in humans, these experimental findings are compatible with presently available information from studies in humans. Other studies are presently in progress to further elucidate the pattern of hemoglobin deposition in haptoglobin-free patients.

Summary

Mechanisms of removal of free hemoglobin from the circulating plasma have been studied experimentally in dogs, employing Cr^{51} labelled hemoglobin.

Free hemoglobin combined primarily with available circulating haptoglobin and this molecule presumably was removed from the plasma by the reticulo-endothelial system in the liver and spleen.

Further accumulation of free hemoglobin, beyond the combining power of haptoglobin, resulted in hemoglobinuria, but only when levels of free hemoglobin of approximately 70 mg.% or more were present in the plasma. Even then, the percentage of available free hemoglobin actually excreted by the kidneys was small and the kidneys did not appear to act as effective excretory organs for free hemoglobin.

Most free hemoglobin, above the combining power of haptoglobin, was removed from the plasma by deposition in various body tissues but chiefly in renal cortex. Progressively higher levels of free plasma hemoglobin resulted in a selective proportionate increase in hemoglobin deposition in the kidneys.

Hemoglobin deposited in liver and kidney was remobilized steadily and fell to immeasureable levels by approximately 100 days, as indicated by tissue radio-activity levels.

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