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Plasma Hematin-Binding and Clearance in the Rhesus Monkey.
(30712)

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The binding of hematin to human serum proteins was suggested by Heilmeyer in 1933 on the basis of spectroscopic evidence(1). In 1934 Fairley and Bromfield described a new heme pigment in the plasma of a malaria patient(2), and Fairley later characterized the heme-albumin complex or methemalbumin more fully with studies both *in vivo* and *in vitro*(3-5). In recent years binding of hematin by human serum globulin, as well as by albumin, has been described. This heme-binding globulin has been reported to be an α_2-globulin(6,7) or a β-globulin(8-10). The quantitative and qualitative patterns of hematin-binding by proteins in serum from many lower animals differ significantly from the pattern in human serum(6,7,11). However, in monkeys Fairley observed rapid formation of methemalbumin in plasma when hematin was injected intravenously(5), and studies of plasma heme pigments after hemolytic transfusion reactions in rhesus monkeys demonstrated patterns of hemoglobin-binding and metabolism similar to those in humans(12). In the latter studies significant amounts of methemalbumin were found in the plasma of monkeys after intravascular hemolysis. These facts suggested that the monkey would provide a useful model for the investigation of hematin-binding and metabolism.

The present studies were undertaken to compare protein binding of hematin by human and rhesus monkey plasma and to trace the disappearance of heme-proteins from the plasma of monkeys after intravenous injection of hematin.

Methods. These studies were performed on female rhesus monkeys (*Macaca mulatta*) maintained on a standard monkey chow diet. Hemin was prepared from human erythrocytes by the method of Labbe and Nishida (13) and was recrystallized once. For intravenous administration hemin was dissolved just prior to use in 1% sodium carbonate (pH 11) at a concentration of 5 mg/ml. This alkaline hematin solution was freed of bacteria by filtration through a Millipore® filter[†] with pore diameter of 0.45 μ. In most experiments the animals were anesthetized for hematin injection with phencytidine,[‡] 7.5 to 10.0 mg intramuscularly. In Experiments 1C and 2B anesthetic was not used. Hematin was injected into the saphenous vein. Blood samples were taken periodically without anesthesia from the saphenous vein, or in a few instances the femoral vein, through a #19 needle into a plastic syringe. The blood was placed in a siliconized tube, centrifuged immediately, and the plasma removed. Plasma samples were frozen and studies performed within a few days after collection. Six hematin-injection experiments were performed in three monkeys (Table I). It will be noted that 2 or 3 weeks elapsed between experiments on the same animal. In Experiment 1C the monkey was given an injection of hematin on each of the 3 days prior to the day of the actual experiment. In Experiment 3A Fe^{59}-hematin was administered.

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[†] Millipore Filter Corp., Bedford, Mass.

[‡] Sernyl®, Parke, Davis and Co., Detroit, Mich.

Total plasma heme pigment levels were determined by the modified benzidine technique of Crosby and Furth(14) using 0.1 M sodium chloride for necessary dilutions. Individual heme-proteins in the plasma were separated by paper electrophoresis in 0.067 M barbital buffer at pH 8.6, stained with benzidine-hydrogen peroxide, and quantified by scanning in a recording photoelectric densitometer§(15). Results are expressed in terms of oxyhemoglobin, which was the standard used for total heme pigment determination. These figures thus reflect the relative amounts of hematin bound to various proteins.

To study plasma protein binding of hematin *in vitro,* hematin (1-2 mg/ml in 1% sodium carbonate prepared just before use) was incubated with plasma for one hour at 37°C. The binding was assessed qualitatively and quantitatively by electrophoresis, benzidine-staining, and photoelectric scanning. The hematin (heme-protein) content of the plasma, expressed in terms of oxyhemoglobin, was determined after incubation by the benzidine method.

Fe^{59}-hematin for Exp. 3A was isolated from erythrocytes obtained by bleeding a rabbit which had been injected one week earlier with approximately 670 microcuries of ferric chloride-Fe^{59} intravenously. Fe^{59}-hematin was injected into the monkey, and blood samples were taken and analyzed as described above. In addition, aliquots of plasma and washed red cells were saved for determination of radioactivity. Twenty-one hours after injection the animal was anesthetized with phenycyclidine and sodium pentobarbital and killed by bleeding from cardiac puncture. The organs were removed immediately and saved for assay of radioactivity. Plasma and red cell samples and organs were placed in 8-ounce plastic bottles and the radioactivity determined in a small animal counter.|| The radioactivity of the remaining carcass was measured in a human whole body counter.¶ The radioactivity of the Fe^{59}-hematin solution injected into the animal was similarly determined in the two counters used. Organ radioactivity was then calculated as a percentage of administered radioactivity.

Results. When human plasma was incubated with hematin and subjected to paper electrophoresis, 2 distinct bands appeared on the benzidine-stained strips. These corresponded to β-globulin and albumin. No free hematin (which remains at the origin under these conditions) was seen. With concentrations of added hematin in the range of 30-60 mg/100 ml, generally 10-20% was bound to β-globulin, while the remainder was bound to albumin to form methemalbumin. Monkey plasma showed a similar pattern of hematin-binding *in vitro* with clearly-defined heme-globulin and methemalbumin bands. In 8 monkey plasmas incubated with 44 to 59 mg/100 ml hematin, 14-26% of the pigment was bound to β-globulin. In addition to the distinct β-globulin and albumin bands, there was frequently a lighter, more diffuse band of benzidine-staining material in the α-globulin region. Some monkey plasmas contained significant amounts of hemoglobin as a result of traumatic venipunctures. In such cases a benzidine-positive band of haptoglobin-bound hemoglobin appeared between the β and α_2-globulin regions, separate from the heme-globulin bands. Similar patterns of binding were seen in plasma samples taken from monkeys after intravenous hematin administration. In Fig. 1 is reproduced an actual densitometer scan of a benzidine-stained paper strip on which monkey plasma obtained after hematin injection was electrophoresed. A sketch of the stained strip is superimposed. The scan shows that the various components were readily separated and quantified by this technique. Rapid intravenous injection of alkaline hematin in the amounts given produced no apparent ill effects in the monkeys. In a pilot experiment, not reported here, an animal was given a dose of 8 mg/kg also without untoward effects.

Fig. 2 shows the time curve of disappearance of hematin from the plasma in the 6 experiments. Fig. 3 and 4 demonstrate the disappearance of the 2 major heme-protein com-

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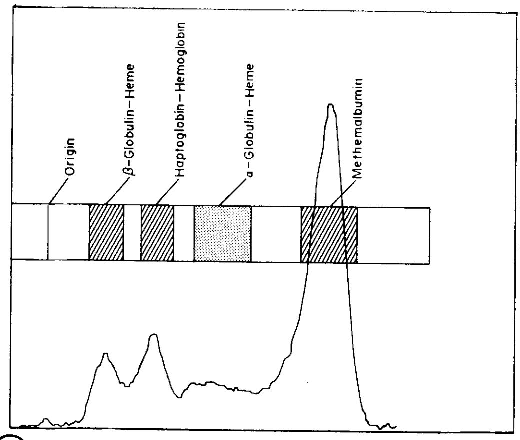
§ Spinco Analytrol, Spinco Division, Beckman Instruments, Inc., Belmont, Calif.

|| Armac Scintillation Detector, Model 440, Packard Instrument Co., Inc., La Grange, Ill.

¶ Liquid Scintillation Spectrometer, Model 11, Packard Instrument Co., Inc., La Grange, Ill.



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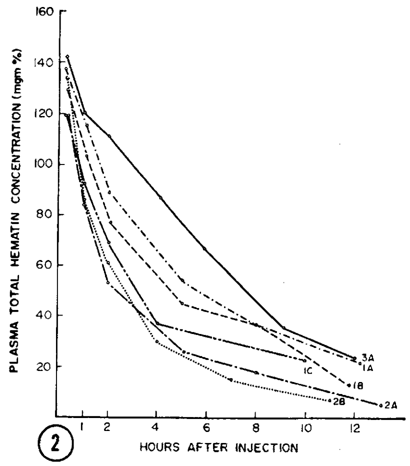

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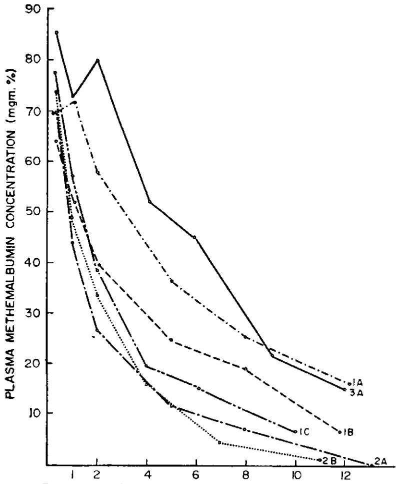

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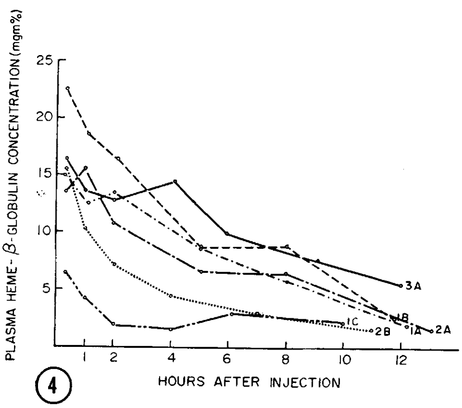

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FIG. 1. Paper electrophoresis of monkey plasma after intravenous hematin injection. Sketch of benzidine-stained paper strip with its densitometer scan.

FIG. 2. Plasma total hematin concentration after intravenous hematin injection.

FIG. 3. Plasma methemalbumin concentration after intravenous hematin injection.

FIG. 4. Plasma heme-β-globulin concentration after intravenous hematin injection.

ponents, methemalbumin and heme-β-globulin. Plasma levels of total hematin and methemalbumin during the period from 1 to 12 hours after injection declined at an approximately exponential rate. The half-lives in plasma derived from such plots are given in Table I. In contrast the disappearance of heme-globulin (Fig. 4) was not exponential and occurred at a slower rate.

When Fe^{59}-hematin was administered (Exp. 3A), the disappearance of total hematin from the plasma could be followed both by the usual method and by the decline in plasma radioactivity (Fig. 5). Statistical comparison of the 2 methods of determining total plasma hematin levels showed close agreement with a correlation coefficient of 0.995. The radioactivity of the animal's organs 21 hours after injection of Fe^{59}-hematin is shown in Table II.

Plasma samples obtained 8 to 30 hours after hematin injection and incubated with additional hematin *in vitro,* demonstrated a generally diminished binding of hematin to β-globulin in comparison to control plasmas. Data illustrating this effect in plasma samples

TABLE I. Description of Hematin-Injection Experiments. Plasma clearance of total hematin and methemalbumin.

Exp	Monkey	Wt (kg)	Hematin administered (mg/kg body wt)	Date of exp	Half-life in plasma (hr)‡ Total hematin	Methemalbumin
1A	D089	4.1	2.6	2-22-65	4.8	5.0
1B	"	4.1	2.6	3- 8-65	3.6	3.6
1C	"	4.1	2.4*	3-31-65	2.2	1.9
2A	D101	3.2	2.5	3- 1-65	3.0	2.6
2B	"	3.2	3.0	3-22-65	2.3	1.8
3A	D099	3.8	2.5†	3-15-65	4.6	4.9

* On the 3 days prior to this experiment the monkey received intravenous injections of 6.1, 9.8, and 9.8 mg hematin per kg body weight, respectively. Hemin was dissolved in 1% sodium carbonate at a concentration of 10 mg/ml for these injections. Unrecrystallized hematin was used for the latter 2 doses.

† Fe^{59}-hematin (unrecrystallized).

‡ The half-lives were derived from semi-log plots of plasma levels against time for the period from 1 to 12 hours after hematin injection except in Exp 1C where data for only the first 4 hours after injection were used.

drawn 21-22 hours after injection are presented in Table III. In Exp. 1C daily injections of hematin were given for the 3 days prior to the experiment to ascertain whether hematin-binding to β-globulin would be further depressed. The low levels of heme-globulin in the animal's plasma after hematin injection are apparent (Fig. 4), and *in vitro* incubation of hematin with post-injection plasma samples demonstrated that hematin-binding to β-globulin was still depressed below control values 48 hours after intravenous hematin administration.

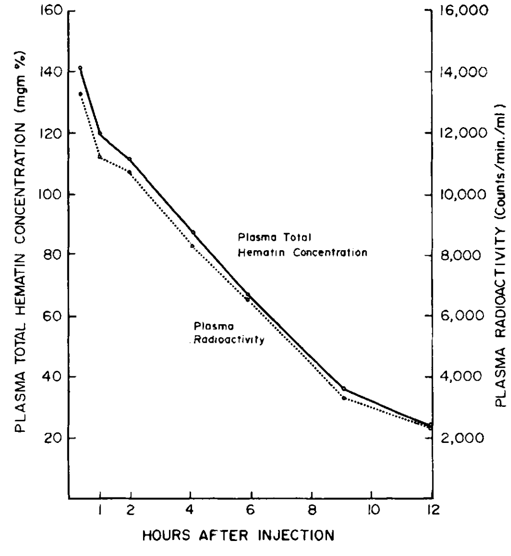

FIG. 5. Plasma total hematin concentration and radioactivity after intravenous Fe^{59}-hematin injection.

TABLE II. Organ Radioactivity 21 Hours After Intravenous Injection of Fe^{59}-Hematin.

Organ	% of injected radioactivity
Liver	48.2
Spleen	.5
Lumbar vertebrae	1.3
Sternum	.2
Kidneys	1.6
Lungs	1.2
Heart	.4
Intestine	2.0
Plasma*	4.8
Red cells*	.1
Remainder of animal	34.2
Total	94.5

* Total plasma and red cell radioactivity were estimated by assuming a blood volume of 50 ml/kg body weight(16) and using the peripheral venous hematocrit at time of sacrifice.

Discussion. The pattern of hematin-binding in monkey plasma after *in vitro* incubation with hematin and after intravenous injection of hematin *in vivo* was very similar to that in human plasma incubated with hematin. The only qualitative difference noted was the appearance of benzidine-positive material in the α-globulin region in monkey plasma after hematin injection. This band was less discrete than those of heme-β-globulin and methemalbumin. It is possible that it represents binding of an altered hematin molecule. Alteration of hematin on standing in alkaline solution has been described (17), and we have observed such an α-globulin band after electrophoresis of human plasma incubated with hematin which had stood

TABLE III. *In vitro* Binding of Hematin to β-Globulin in Plasma Before and 21-22 Hours After Intravenous Hematin Injection.

	Heme-globulin (mg/100 ml)	
Exp	Control plasma	Plasma 21-22 hr after hematin injection
1A	11.9 (58.5)	6.8 (57.4)
1B	11.5 (43.7)	7.3 (45.3)
2A	9.5 (56.6)	8.2 (57.2)
2B	10.0 (53.3)	6.9 (55.7)
3A	10.6 (54.5)	5.6 (55.8)

Figures in parentheses indicate amount of hematin (mg/100 ml) added to plasma prior to *in vitro* incubation.

in alkaline solution overnight. Nevertheless, it is clear that of the large number of species that have been studied(6,7,11), the monkey offers the best animal model of hematin-binding and metabolism in man.

The clearance of hematin from the plasma of monkeys after intravenous injection was slower than the clearance of hemoglobulin after intravascular hemolysis in such animals (12). In humans, too, there is evidence to suggest that protein-bound hematin is cleared from the circulation less rapidly than hemoglobin. Methemalbumin has been shown to persist for as long as 2 days in human plasma after hematin injection(4,18), and in patients with chronic intravascular hemolysis the majority of the increase in total plasma heme pigment is commonly due to methemalbumin rather than free hemoglobin(19,20).

In the case of hemoglobin bound to haptoglobin, there is evidence that the haptoglobin-hemoglobin complex is removed as a unit from the plasma by the reticulo-endothelial system(21), and the half-life of haptoglobin in the circulation is shortened considerably when it is complexed with hemoglobin(22). The data presented here do not distinguish between removal of the heme-albumin complex or simply removal of the heme moiety with the return of the albumin to the circulation. If the whole complex is removed, it is clear that attachment of heme to albumin markedly shortens the half-life of that protein in the plasma (assuming that monkey albumin, like human albumin, has a plasma half-life measured in days)(23). If only heme is removed, then albumin functions in this situation as a transfer protein, resembling transferrin which binds and transports iron.

The data of Exp. 3A (Table III) make it clear that the liver is primarily responsible for removal of protein-bound hematin from the plasma. Presumably the hematin is then degraded to bile pigment as has been shown in dogs(24) and suggested in humans(18). It has been demonstrated that the liver is also primarily responsible for the removal of the haptoglobin-hemoglobin complex from the plasma in animals(25,26) and man(27).

In all cases when a monkey was injected with hematin 2 or 3 weeks after a previous injection (Exp. 1B, 1C, and 2B), clearance of hematin from the plasma was more rapid following the second injection. Perhaps the first exposure to hematin served to stimulate the cellular or enzymatic systems responsible for its removal from the plasma. An analogous situation has been described by Shen who observed an accelerated removal of hemoglobin from the plasma of patients with chronic hemolytic anemia(28).

Compared to methemalbumin the relatively slow, non-exponential removal of heme-β-globulin from the circulation may indicate that it was replenished during the experiment by heme removed from other proteins. Heme-binding globulin is reported to have a greater affinity for heme than does albumin(9), and in experiments not reported here we have shown that human β-globulin can "capture" heme from preformed methemalbumin. Undoubtedly a complex relationship exists in the plasma depending on the concentrations of heme and its binding proteins and the relative affinities of those proteins for heme. Nevertheless, the partial depletion of heme-binding globulin following hematin-injection (Exp. 1C and Table III) suggests that the heme-globulin complex may have been removed from the circulation as a unit, rate of removal exceeding rate of production of heme-binding globulin. This protein would then be depleted in a manner analogous to the depletion of haptoglobin by hemolysis. Though little is known about the role of heme-binding globulin *in vivo* in humans, it has been reported to be absent from the serum of patients with hemolytic anemia(8,29), suggesting a process of depletion of the protein through removal of the whole complex from the plasma.

Summary. In vitro and *in vivo* studies demonstrated that protein-binding of hematin in rhesus monkey plasma was similar to that in human plasma. After intravenous injection of hematin in monkeys, the pigment was bound primarily by albumin and β-globulin, and possibly to some extent also by α-globulin. The disappearance of the heme-protein complexes from the plasma was traced, and studies with Fe^{59}-hematin implicated the liver as the primary site of removal of injected hematin. Depletion of the heme-binding globulin was observed after hematin injection. The value of the monkey as an experimental model for studies of hematin-binding and the possible implications for human hemolytic disease are discussed.

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Plasma Hemoglobin Binding and Clearance in the Rhesus Monkey After Hemolytic Transfusion Reactions. (30713)

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Man shares a number of biological properties with other primates(1). Hematologically, for example, non-human primates exhibit similarities to man in morphological and

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