tients 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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TABLE I. Antibody Titers in Monkey Serum Before Transfusion and After Immunization with Human Group A_1 Red Cells.

	Agglutination titer of serum for unmodified human red cells			
Monkey No.	Type O	Type A_1	Type A_2	Type B
665	0	1:14	1:16	1: 1½
666	0	1:30	1:10	0
671	0	1:5	1: 1½	0
779	1:15	1:40	1:20	1:15

antigenic properties of the formed elements of the blood(2-4), patterns of red cell glucose metabolism(5), and amino acid sequences of the peptide chains of hemoglobin(6). In addition the plasma proteins of lower primates correspond closely to human plasma proteins(7). Specifically, monkeys have types of haptoglobin similar to known types in man(8). Because of these similarities, rhesus monkeys were utilized in the present study for investigation of heme pigment metabolism after transfusion reactions. The results indicate that the binding and metabolism of plasma heme pigments are qualitatively similar in man and monkeys. The rhesus monkey therefore serves as a useful model for experimental purposes not practical in man.

Methods. Female rhesus monkeys (*Macaca mulatta*), weighing between 3.5 and 4.4 kg were maintained on a standard monkey chow diet. Phencytidine† was administered intramuscularly in a dosage of 2 to 4 mg/kg as anesthetic agent during the hemorrhage and transfusion. Periodic blood sampling was performed without anesthesia. The ABO (H) blood groups of the monkeys were determined by the method of Wiener *et al*(9). In the 4 monkeys utilized for the transfusion experiments, anti-A antibodies were present in the serum, and blood group B substance was detected in the saliva. They were thus classified as having "human-like" blood group B. Neither anti-O nor anti-B antibodies were present in the serum before the animals were immunized with human group A_1 red cells. Anti-O and anti-B antibodies detected in the serum of some animals after such immunization demonstrated the appearance of heterohemagglutinins (Table I).

† Sernyl®, Parke, Davis & Co., Detroit, Mich.

Severe hemolytic reactions were obtained in the rhesus monkeys by transfusion of human group A blood after a preliminary course of sensitization with A antigen: the monkeys were given an initial intravenous infusion of human group A_1 blood, approximately 6 ml/kg, and after an interval of 3½ months an intravenous booster dose of 5 ml of blood from the same donor. Two weeks later the serum antibody titers were determined (Table I), and the animals were transfused with the same group A_1 donor blood which had been stored for 2 weeks at 4°C in acidified citrate dextrose. Animals were bled of 20% of their estimated blood volume (1% of body weight) and were maintained in this hypovolemic state for 2 hours. Blood pressure in the femoral artery was continuously monitored by means of a siliconized vinyl cannula connected to a mercury manometer. Then during a 30-minute period they were transfused with the group A_1 human blood in volumes up to 16% of their estimated blood volume. Following transfusion the animals were kept in metabolic cages, and urine was collected and tested for hemoglobin.

Blood samples were taken prior to the initial hemorrhage and at intervals following completion of the transfusion. Blood was obtained by venipuncture of the femoral or saphenous vein using a #19 or #20 needle and a plastic syringe. The blood was placed in a siliconized tube containing a drop of heparin, centrifuged immediately, and the plasma removed. Plasma samples were stored in the frozen state and the studies performed from 1 to 28 days after collection. Results were constant during this period of storage.

Total heme-pigment levels were determined by the modified benzidine method of Crosby and Furth(10) using 0.1 M sodium chloride for any necessary dilutions. In the plasma of the transfused monkeys the several heme pigments (free hemoglobin, haptoglobin-bound hemoglobin, and methemalbumin) were separated by paper electrophoresis in 0.05 M phosphate buffer at pH 7.0. The paper strips were stained with benzidine-hydrogen peroxide and the heme pigment bands quantified by scanning in a recording photoelec-

TABLE II. Haptoglobin Levels in Monkey Plasma. Comparison of binding capacity for monkey and human hemoglobin.

Plasma	Hemoglobin binding capacity (mg/100 ml)	
	Autologous monkey hemoglobin	Human hemoglobin
Monkey 665	72	71
" 666	68	72
" 671	43	42
" 779	100	95

tric densitometer‡(11). All results are expressed in terms of oxyhemoglobin, which was the standard used in the total heme pigment determination.

Plasma haptoglobin levels (hemoglobin-binding capacity) were determined electrophoretically after incubation of plasma for one hour at 37°C with known amounts of human or monkey hemoglobin(11). Human and monkey hemoglobin solutions, containing 4-6 g of hemoglobin per 100 ml were prepared by hemolyzing washed erythrocytes with water, removing the stroma by centrifugation, and dialyzing the clear supernatant solution thoroughly in the cold against 0.1 M sodium chloride. The hemoglobin content of these solutions and of the standards used for the total heme pigment determinations was measured by the cyanmethemoglobin method(12).

Protein patterns in monkey and human plasma, before and after incubation with hemoglobin, were compared by paper electrophoresis in 0.067 M barbital buffer at pH 8.6. Some paper strips were stained for protein with bromphenol blue, others for heme pigments with benzidine-hydrogen peroxide.

Results. The haptoglobin levels in plasma specimens from 4 monkeys were determined using monkey and human hemoglobin (Table II). In each case the hemoglobin-binding capacity was the same with either type of hemoglobin. (Conversely, human plasma also bound identical amounts of human and monkey hemoglobin.) Therefore, human hemoglobin was used for subsequent determination of haptoglobin levels in monkey plasma. Haptoglobin levels, determined in ten monkeys, ranged from 42 to 123 mg/100 ml hemoglobin-binding capacity with a mean value of 69 mg/100 ml.

Electrophoresis at pH 8.6 revealed monkey plasma to have a less distinct β-globulin band and a more prominent α_2-globulin band than human plasma. After incubation of the plasmas with hemoglobin, the α_2-band was diminished in intensity in both monkey and human plasma, and a new band, which stained with benzidine, appeared between the β and α_2 regions. These findings indicate that monkey haptoglobin, like its human counterpart, is an α_2-globulin and that the monkey haptoglobin-hemoglobin complex, also like that in humans, migrates between β- and α_2-globulins under these electrophoretic conditions.

Infusion of group A_1 human blood into the monkeys, which had been previously immunized with blood from the same donor, produced severe hemolytic transfusion reactions. Hemoglobinuria occurred in all animals except #779. The severity of reaction was related to the antibody titer found in the serum prior to the final transfusion (Table I). Monkey #779, the only one in this group with a high heterohemagglutinin titer (anti-O and anti-B), became acutely hypotensive and died shortly after infusion of only 10 ml of incompatible blood. Monkey #666, which had a high anti-A_1 titer, became very hypotensive after infusion of 11 ml, and the transfusion was stopped. The other 2 monkeys, #665 and #671, received 38 and 37 ml of incompatible blood, respectively, and had milder reactions, consisting mainly of respiratory wheezing, prolonged expiration, and pulmonary rales. In addition #671 became somewhat hypotensive for about 30 minutes. Clinical, serological, and hemodynamic aspects of these transfusion reactions will be reported separately(13,14). Plasma heme pigment levels in the 2 animals which received full transfusions are shown in Tables III and IV. The time required for disappearance of half the total heme pigment from the plasma of monkeys #665 and #671 was 130 and 115 minutes, respectively, and half the free hemoglobin had disappeared from

‡ Spinco Analytrol, Spinco Division, Beckman Instuments, Inc., Belmont, Calif.

TABLE III. Plasma Heme Pigment Levels After Infusion of Incompatible Blood to Monkey #665.

Time after infusion	Total heme pigment	Free hemoglobin	Haptoglobin-bound hemoglobin	Methemalbumin
Control	2.4		2.4	
7 min	801	729	43	29
1 hr 50 min	487	393	30	64
4 "	244	143	36	65
8 " 30 min	133	35	35	63
26 " 30 "	37.4	3.1	28.1	6.2
51 "	1.2		1.2	

Blood was infused over a 30-min period, and samples are timed from completion of the infusion. All figures are expressed in mg/100 ml (relative to oxyhemoglobin).

TABLE IV. Plasma Heme Pigment Levels After Infusion of Incompatible Blood to Monkey #671.

Time after infusion	Total heme pigment	Free hemoglobin	Haptoglobin-bound hemoglobin	Methemalbumin
Control	2.2		2.2	
8 min	666	575	28	63
2 hr	316	214	31	71
4 "	159	98	31	30
9 "	52.5	5.9	21.9	24.7
26 " 30 min	5.4		3.2	2.2
51 "	3.8		3.8	

Blood was infused over a 30-min period, and samples are timed from completion of the infusion. All figures are expressed in mg/100 ml (relative to oxyhemoglobin).

the plasma in 110 and 85 minutes, respectively.

As is apparent from Tables III and IV, haptoglobin never completely disappeared from the plasma following the acute hemolytic episodes. Total plasma haptoglobin levels during the 2 weeks following transfusion reactions are plotted for 3 animals in Fig. 1.

Discussion. Reich showed that serum from one species of animal bound hemoglobin from another and that the patterns of binding were characteristic of the serum rather than the added hemoglobin(15). Our studies have demonstrated that human and monkey plasma bind the same quantity of hemoglobin from either species and that haptoglobin-hemoglobin complexes of both species have similar electrophoretic mobilities.

Starch gel electrophoresis of human serum has led to the description of 3 common haptoglobin phenotypes, 1-1, 2-2, and 2-1(16). Studies of monkey serum have generally shown haptoglobin patterns like the human

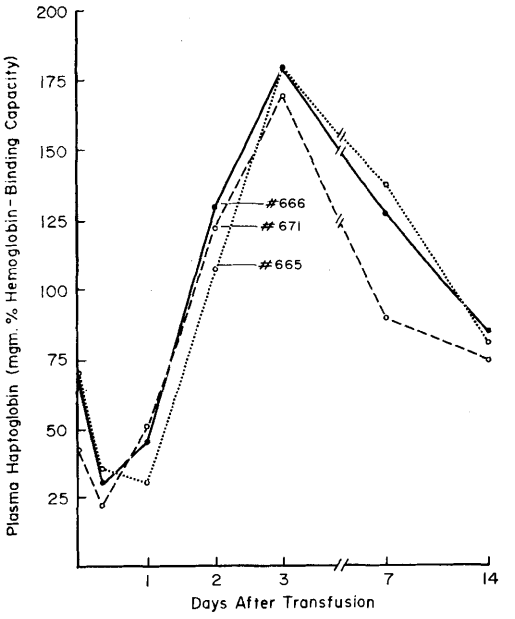

FIG. 1. Plasma haptoglobin levels following hemolytic transfusion reactions in 3 rhesus monkeys.

type 1-1(8,17-19), though a pattern similar to 2-1 has been described(20). In man the total amount of haptoglobin is related to the phenotype(21). Haptoglobin types were not determined in the animals studied here, but the mean hemoglobin-binding capacity of about 70 mg/100 ml is below that reported for any of the phenotypes in humans. Since haptoglobin is quantified only in terms of its functional capacity to bind hemoglobin, these quantitative differences may reflect either a difference in hemoglobin-binding capacity of each haptoglobin molecule or, more likely, a difference in concentration of haptoglobin molecules in the plasma.

Plasma clearance of the haptoglobin-hemoglobin complex has been investigated extensively in animals and man, and the kinetics have been variously described(22). Less information is available, however, about the clearance of free hemoglobin from the plasma. Murray *et al*(23) reported a half-life of 25 minutes for plasma free hemoglobin in rabbits. Lathem and Worley(11) observed that clearance of free hemoglobin from human plasma occurred at an exponential rate with a half-life of 207 minutes. In the monkeys in this study the free hemoglobin disappearance best fits an exponential plot, at least up to 9 hours, with half-times in the plasma of 85 and 110 minutes. The decline of total heme pigment in the plasma obviously reflected several concurrent processes: removal of free hemoglobin and haptoglobin-hemoglobin complexes, synthesis or mobilization of haptoglobin, and formation and removal of methemalbumin. For the first 4 hours total heme pigment disappearance also occurred at an exponential rate (with half-times of 115 and 130 minutes), undoubtedly due to the predominant influence of the high levels of free hemoglobin present in the period immediately following transfusion.

The failure of haptoglobin to disappear completely from the plasma despite the large amounts of hemoglobin released as a result of the intravascular hemolysis suggests that it was rapidly synthesized or mobilized from extravascular sites. The stimuli to haptoglobin synthesis are poorly understood. Since it is absent from the plasma in most patients with hemolytic anemia(24), its depletion by hemolysis has been regarded as a rather weak stimulus to its synthesis(25). On the other hand, inflammation and tissue necrosis seem to be potent stimuli to haptoglobin generation(21,25). In the monkeys studied here autopsies 2 weeks after the transfusion reactions showed no tissue necrosis. However, in other animals in which the transfusion reaction was immediately fatal, autopsy frequently revealed areas of hepatic and splenic necrosis. The marked "rebound" increase in plasma haptoglobin following the transfusion reactions to levels well above the baseline values reflects a response to stimuli as yet undefined. Such an increase has been noted after surgery and has been described in 2 human subjects following transfusion reactions(26). The rate of disappearance of "excess" haptoglobin 3 to 14 days after transfusion reaction can be used as a crude estimation of the half-life of haptoglobin. In our studies the T ½ of this "excess" haptoglobin was 3.2 to 5.5 days. Using similar calculations in patients recovering from pneumonia, Nyman found a T ½ of the "excess" haptoglobin of about 5 days(21) and Jayle and Moretti reported the T ½ of I^{131}-labelled haptoglobin in 3 human subjects to be 2.5 to 5 days(16).

Methemalbumin is commonly found in the plasma of patients suffering from intravascular hemolysis(27). It is formed during incubation of heme with the plasma of some lower animals(28) and has been identified in monkey plasma after incubation *in vitro* with heme(29) and *in vivo* after intravenous hematin injection(30). Our studies demonstrated its formation in monkey plasma from hemoglobin released by intravascular hemolysis and its persistence beyond 24 hours. The fact that methemalbumin levels remained stable or fell only slowly while hemoglobin was being rapidly cleared from the plasma suggests that it was being continually formed or that its clearance was slower than that of free or haptoglobin-bound hemoglobin(31).

It is apparent from these studies of rhesus monkeys that the plasma binding of heme pigments *in vitro* and *in vivo* following hemolytic transfusion reactions is remarkably simi-

lar to that in humans. Coupled with the known similarities of blood groups and hemoglobin and haptoglobin structure in the two species, these facts recommend the monkey as an experimental animal for the study of hemolytic transfusion reactions and other forms of intravascular hemolysis.

Summary. Binding of hemoglobin by rhesus monkey plasma was studied *in vitro* and after hemolytic transfusion reactions *in vivo*. Monkey haptoglobin was found to bind the same amount of monkey or human hemoglobin and was very similar to human haptoglobin in its electrophoretic characteristics. The mean plasma haptoglobin level in 10 monkeys was 69 mg/100 ml. Following hemolytic transfusion reactions, free hemoglobin, haptoglobin-bound hemoglobin, and methemalbumin were identified in the plasma, and their disappearance was traced. The value of the rhesus monkey as an experimental animal for the study of intravascular hemolysis and plasma hemoglobin binding and metabolism is stressed.

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