FATE OF LABELLED HAEMOGLOBIN IN NORMAL AND *PLASMODIUM COATNEYI*-INFECTED MONKEYS†

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INTRODUCTION

The mechanism of haemolysis in malaria infection is still not clear at the present time. It has been assumed that both parasitized and non-parasitized cells were destroyed either by intravascular haemolysis or by erythro-phagocytosis. Haemoglobin released into plasma usually bound itself firmly to the haptoglobin to form complexes, the physical properties of which were not fully defined. It has been reported that the binding of haemoglobin to haptoglobin had little effect *in vivo* on the rate of conservation to bile pigments (Ostrow *et al.*, 1962) or on the rate of re-utilization of haemoglobin iron (Murray *et al.*, 1961). In studies with large amounts of haemoglobin injected repeatedly into normal healthy animals, most of the iron of free haemoglobin was recovered in the kidney and the remainder was observed to be deposited in the liver, spleen and bone marrow (Finch *et al.*, 1950). A study in normal rats receiving single injections of small amounts of ^{59}Fe-haemoglobin indicated the liver to be the major site of haemoglobin deposition (Garby and Obara, 1960).

The fate of the haemolysed red cells and subsequently of the liberated haemoglobin prior to its degradation in malaria infection is not known. Reticulo-endothelial cells have generally been held responsible for the breakdown of haeme moiety of the haemoglobin molecule. The chief organ site of haemoglobin breakdown is unknown, although liver, spleen and bone marrow have been shown to produce pigments.

The present study was an attempt to investigate the fate of labelled haemoglobin in monkeys infected with *Plasmodium coatneyi* in comparison with normal monkeys by injecting intravenously ^{51}Cr-labelled haemoglobin to simulate intravascular haemolysis.

MATERIALS AND METHODS

All experiments were performed on rhesus monkeys *(Macaca mulatta)* weighing between 2.0 and 4.6 kg., anaesthesized with intravenous injection of Sodium pentobarbital (10-15mg/kg). Seven normal monkeys and 4 monkeys infected with *P. coatneyi* were used in the study.

Infected monkeys : Monkeys were infected with *P. coatneyi* from a donor monkey by a method described previously (Areekul *et al.*, 1971 a). Haemoglobin, haematocrit, red blood cell count and parasitaemia in thick and thin smears of blood were recorded daily. The study of haemoglobin metabolism was initiated only when the haemoglobin and haematocrit values decreased to about half of the initial value, usually about 20 days after inoculation of the infected blood.

Preparation of labelled haemoglobin solution: 10-15 ml of the experimental subjects's own blood was labelled with 50-80 μCi of ^{51}Cr by the usual method (Veall and Vetter, 1958). Haemoglobin solutions were prepared from

† This work was supported by research grant, No. 674/R2/RB from the International Atomic Energy Agency, Vienna, Austria.

the labelled red cells by adding distilled water and thawing. The haemolysates were centrifuged at 1500 rpm for 5 minutes and the supernatants were diluted with normal saline solution. The final solutions used for injection into the recipients contained 6.9 to 10.6 gm per 100 ml of haemoglobin and 3-11 $\times 10^5$ cpm per ml of ^{51}Cr. The amount of haemoglobin given was 116 to 500 mg per kg. body weight. Aliquots of the solution were taken and counted with the samples. The haemoglobin was determined by the cyanmethaemoglobin method.

Estimation of radioactivity in plasma and internal organs

^{51}Cr-labelled haemoglobin solution was injected intravenously into each experimental monkey. The radioactivity in the plasma was measured at 10, 20, 30, 60, 90, 120 minutes, 4, 6 and 24 hours after injection. The activity over the liver, spleen, bone marrow and urinary bladder were recorded by external counting at $\frac{1}{2}$, 2, 4, 6, 24 and 48 hours after injection of labelled haemoglobin. The results were calculated as excess counts and expressed as cpm according to the method of Veall and Vetter (1958).

Calculation of the clearance rate of haemoglobin from the plasma

It is assumed that the plasma haemoglobin compartment represents a pool with a concentration, C, of haemoglobin in a volume, V, of plasma. A large amount of haemoglobin solution is injected intravenously and the decrease in concentration of C is proportional to the concentration at that moment (cf. results below). This process corresponds to a first order reaction, i.e.

$$\frac{dC}{dt} = - kC \qquad \ldots (1)$$

The amount of eliminated material is obtained from:-

$$V \cdot \frac{dC}{dt} = - kVC = J_{out} \qquad \ldots (2)$$

$$\text{or} \qquad J_{out} = \frac{(C_{t_1} - C_{t_2}) \cdot V}{t_2 - t_1} \qquad \ldots (3)$$

where J_{out} is the outflow of haemoglobin from the plasma, and C_{t_1} and C_{t_2} are the haemoglobin concentrations in the plasma at time t_1 and t_2 respectively. The concentrations in these experiments were calculated as the average concentrations from :-

$$C = \frac{C_{t_1} + C_{t_2}}{2} \qquad \ldots\ldots (4)$$

The values of C were obtained from the number of cpm found in the plasma and the known number of cpm per mg haemoglobin injected. Plasma volume was calculated on an assumption that an average plasma volume of the normal monkeys and infected monkeys were 40.5 ml/kg and 50.3 ml/kg respectively (Miller *et al.*, 1968).

RESULTS

The weight, haemoglobin, haematocrit values and the amount of injected haemoglobin are shown in Table 1. The results of the measured plasma haemoglobin radioactivity as a function of time are shown in Fig. 1. It appeared from the figure that the disappearance of the radioactivity followed relatively closely a first order reaction over the range that was observed. In the normal group, the plasma haemoglobin half-disappearance time ($T_{\frac{1}{2}}$) was found to be, on average, 102 minutes (range 75-136 minutes). The radioactivity left in the plasma at 6 hours after injection was 6-13% of the administered dose and at 24 hours was 3-4% of the dose. In the infected group, the average $T_{\frac{1}{2}}$ was found to be 82 minutes (range 63-125 minutes) which was much lower than in the normal group.

Table 1

The amount of injected haemoglobin and the half-disappearance time ($T_{\frac{1}{2}}$) in normal and infected monkeys.

No	Wt (kg)	Sex	Hb (gm%)	Hct (%)	Amount of Hb injected				$T_{\frac{1}{2}}$ (min)	Parasite per 1000 RBC
					gm%	ml	mg	mg/kg		
Normal monkeys										
Pk-32	3.8	F	11.2	32	8.1	8	648	171	97	-
Pk-34	4.6	F	11.7	38	7.3	16	1260	272	88	-
Pk-35	2.9	M	12.2	39	8.6	12	1030	353	93	-
Pk-36	3.0	F	10.3	32	7.3	11	803	267	126	-
Pk-37	3.9	M	12.5	43	10.6	10	1060	272	102	-
MS-94	2.0	F	11.2	38	10.0	10	1000	500	136	-
MS-96	3.0	F	10.0	40	9.4	10	940	313	75	-
Mean	3.3	-	11.3	32	8.7	11	963	307	102	-
Infected monkeys										
Pk-29	4.0	M	5.5	20	7.8	9	702	176	68	20.0
Pk-34	4.6	F	6.9	26	8.9	10	890	194	63	1.0
Pk-35	4.5	M	6.0	20	6.9	10	690	153	125	43.0
Pk-19	4.3	F	4.1	16	7.1	7	497	116	73	41.0
Mean	4.3	-	5.6	21	7.6	9	694	160	82	-

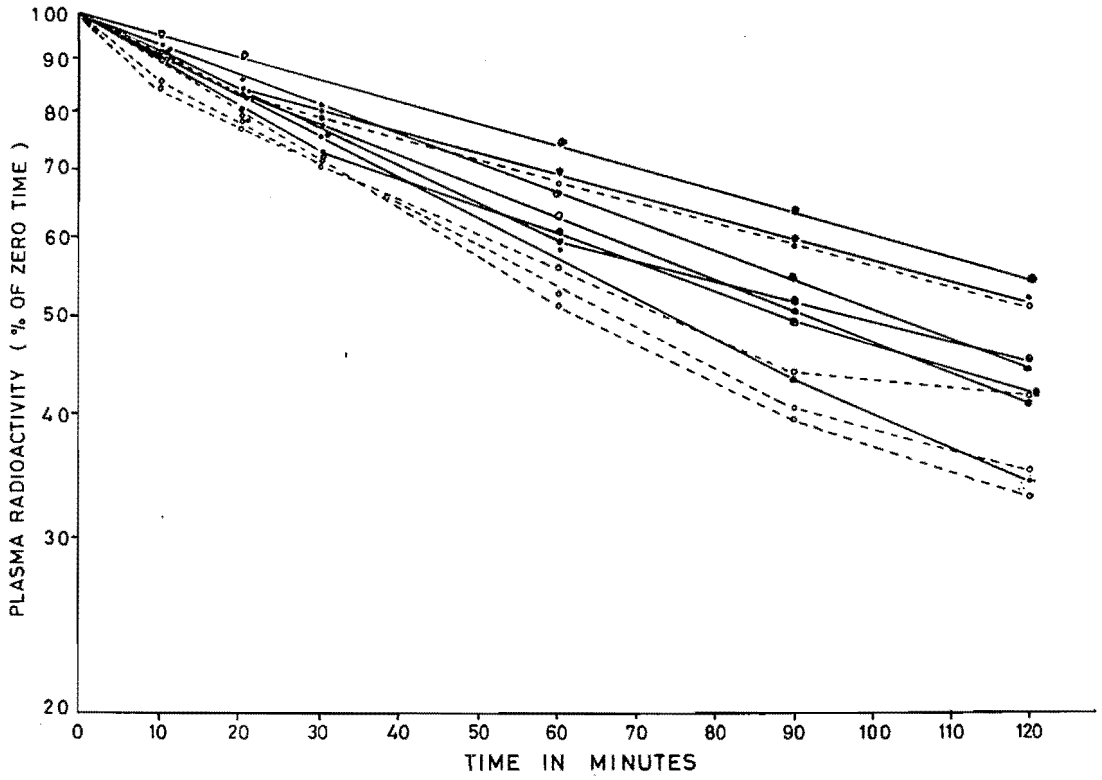

Fig. 1—The time course of the radioactivity from ^{51}Cr-labelled haemoglobin injected at zero time. •——• = normal monkeys, o- - - - o = infected monkeys.

The clearance rate of haemoglobin from the plasma

In Fig. 2, the outflow of haemoglobin (J_{out}) is plotted against the concentration C, calculated from expressions (3) and (4) respectively, in which the data of Sears and Huser (1966) was included. The figure indicates a direct relationship between the outflow and the plasma haemoglobin concentration. This relationship was also shown in the infected monkeys (Fig. 3) but the clearance rate of haemoglobin from the plasma was higher in the infected monkeys than in the normal group at any given levels of haemoglobin concentration.

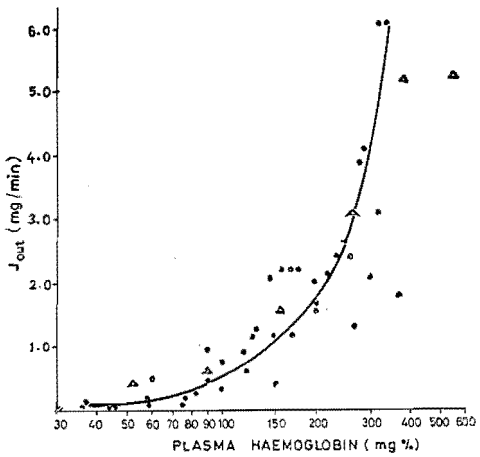

Fig. 2—The relation between the flow of haemoglobin out of plasma, J_{out}, and the plasma haemoglobin concentration. △ = from data of Sears and Huser (1966), • = from present experiments.

Radioactivity in the organs

Normal group : Radioactivity over the liver and spleen rose rapidly during the first few hours after the injection and reached a maximum within 4 hours, then remained at about the same level or decreased slightly for 24 or 48 hours (Fig. 4). Counts over bone marrow were raised in some cases but relatively less than over the liver and spleen. Very high radioactivity was detected over the urinary bladder at 6 hours but none could be detected at 24 hours.

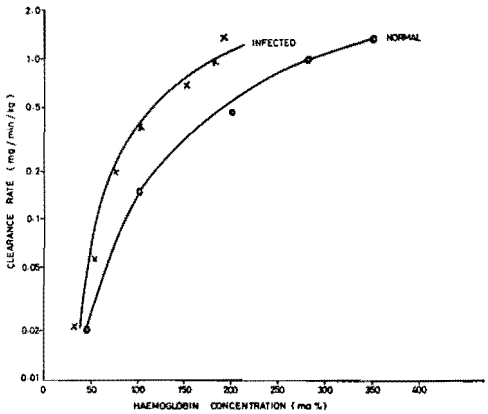

Fig. 3—The relation between the mean clearance rate of haemoglobin from plasma (mg/min/kg) and the plasma haemoglobin concentration (mg%) in normal and infected monkeys.

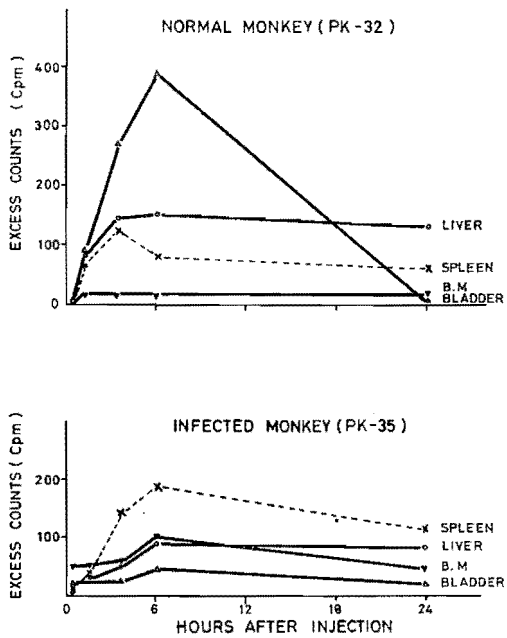

Fig. 4—The radioactivity surface counts over the liver, spleen, bone marrow and bladder in normal and infected monkeys.

Infected group : The results were similar to those observed in the normal monkeys. No significant difference in the radioactivity of the organs was observed between the infected and normal monkeys.

Radioactivity in the urine

Urine was collected from 3 normal and 2 infected monkeys. The results of the accumulated urinary excretion of radioactivity, calculated as a percentage of the given dose, are shown in Table 2.

Table 2
The accumulated urinary excretion of radioactivity, calculated as a percentage of the given dose.

Mean values	Time in hours			
	24	36	48	72
Three normal monkeys (Pk-37, Ms-94, Ms-96)	20.3	21.3	23.1	26.6
Two infected monkeys (Pk-19, Pk-35)	25.9	30.1	32.6	39.8
Per cent elevation over normal	27.6	41.2	41.1	49.6

DISCUSSION

In the present experiments the half-disappearance time in normal monkeys was found to be, on average, 102 minutes (range 75-136). The results were in accordance with data obtained by Sears and Huser (1966) who found the half-disappearance time to be 85 and 110 minutes in two normal monkeys after haemolytic transfusion reaction. In a previous study in monkeys infected with *P. coatneyi* with a tracer amount of ^{59}Fe-haemoglobin, the half-disappearance times were found to be 85, 105 and 163 minutes in 3 normal monkeys (Areekul *et al.*, 1971 b). These results indicated that in normal monkeys, the haemoglobin cleared very rapidly from the circulation.

A large amount of haemoglobin was injected intravascularly in the present experiments and, at 24 hours after injection, only about 3-4% of the injected haemoglobin was found to be left in the circulation while 20% was excreted in urine in the normal monkeys. When such a large amount of haemoglobin was given intravenously, some of it was bound to haptoglobin in the blood to form complexes (Sears and Huser, 1966). This union inhibited haemoglobin from escaping through the glomerulus, thus conserving metabolites as well as preventing renal tubular injury. Haemoglobin which remained in the free form was excreted in the urine or taken up by the liver, spleen and bone marrow as indicated by the radioactivity recorded over these organs in the present experiments. In earlier studies with large amounts of haemoglobin injected repeatedly into animals (Newman *et al.*, 1932; Finch *et al.*, 1950), much of the iron of free haemoglobin was also observed to be deposited in the liver, spleen and bone marrow.

In this study, the results showed that the disappearance rates of haemoglobin from the plasma in the normal monkeys were proportional to the levels of haemoglobin concentration when the concentrations were over 70 mg%. This value corresponded with the haptoglobin levels, estimated in 10 normal monkeys, which were found to be 42 and 123 mg% haemoglobin binding capacity with a mean value of 69 mg% (Sears and Huser, 1966). This suggested that the rate of outflow of haemoglobin from the plasma was related to its concentration when the amount of haemoglobin in the plasma exceeded the binding capacity of haptoglobin. These findings were in agreement with data obtained from humans which demonstrated a constant outflow of haemoglobin from the plasma for various plasma haemoglobin concentrations not exceeding the plasma haemoglobin binding capacity (Garby and Noyes, 1959). When the plasma haemoglobin concentration

was over 100 mg%, i.e. over the binding capacity of haptoglobin, there was a relationship between the plasma disappearance rates and the plasma haemoglobin concentrations (Gilligan *et al.*, 1941).

The half-disappearance time in monkeys infected with malaria was found to be shorter than in normal monkeys (Table 1). This finding was confirmed by the calculated clearance rate of haemoglobin from the plasma. The results showed that the clearance rates in the infected monkeys were approximately 1.3 times higher than in normal monkeys (Fig. 3). Part of the higher disappearance rate in the infected monkeys may be due to the excretion of haemoglobin in urine which was found to be 27 and 50% higher than normal monkeys at 24 and 72 hours respectively. It has been shown that the haptoglobin levels in persons with haemolytic anaemia were low or absent from serum (Nosslin and Nyman, 1958). In a continuing haemolytic process, represented by malaria infection in the present experiments, a large amount of haemoglobin released by red blood cell destruction will have been bound to the haptoglobin, thereby reducing the levels of haptoglobin in the plasma. Most of the injected labelled haemoglobin in the infected monkeys was therefore in excess of the binding capacity of haptoglobin and subjected to excretion by the kidneys. These findings were in accordance with a previous report (Areekul *et al.*, 1971 b) which showed that the half-disappearance times of ^{59}Fe-labelled haemoglobin injected as a tracer dose in monkeys after malaria infection were much shorter than before infection.

SUMMARY

^{51}Cr-labelled haemoglobin was used to study the fate of haemoglobin in rhesus monkeys *(Macaca mulatta)* infected with *P. coatneyi* in comparison with non-infected monkeys. Plasma disappearance rate, radioactivity over the liver, spleen, bone marrow and urinary excretion of labelled haemoglobin were observed up to 72 hours after intravenous injection. It was found that the infected monkeys excreted more haemoglobin in the urine than the normal monkeys and the disappearance rates of haemoglobin from the plasma of the infected monkeys were also faster than in the normal monkeys. The calculated clearance rate of haemoglobin from the plasma in the infected monkeys was about 1.3 times higher than the normal group. There was no significant difference of the radioactivity recorded over the liver, spleen and bone marrow between these two groups of monkeys.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Curt R. Schneider for reading and criticizing the manuscript and Professor Chamlong Harinasuta, Dean of the Faculty of Tropical Medicine, for his support.

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