platelets in citrated platelet-rich plasma even in dilutions of 1:100. The effect is unrelated to blood clotting. The active material in human subcutaneous tissue is sedimented at 25,000 *g* in one hour, stable at 56°C, but not at 70°C, and destroyed by collagenase, formamide and 25% alcohol but not by proteolytic enzymes. An inhibitor is present in liver. Similarities and differences between the effects of purified collagen, tissue suspensions and thrombin on platelets are described and the probable role of the connective tissue factor in hemostasis is discussed.

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Kinetics of Plasma Hemoglobin Catabolism in the Rat. I. Blood Clearance of I^{131} Tagged Hemoglobin.* (27336)

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Despite a vast accumulation of experimental data, the precise mechanism by which circulating hemoglobin is cleared from the blood is still undetermined. Whipple's classic theory of a "renal threshold" claimed that excretion by the kidneys occurred only when the plasma hemoglobin level exceeded this "threshold". Laurell and Nyman(1) subsequently hypothesized that in humans the hemoglobin binding capacity of the plasma haptoglobin is actually the "renal threshold". Hemoglobin, combined with haptoglobin, they maintained, is removed and degraded, presumably by the reticulo-endothelial system, while unbound hemoglobin in excess of haptoglobin is rapidly excreted by the kidneys. This appealingly simple concept, although confirmed in humans by Lathem(2), was not apparent when the clearance of injected hemoglobin was investigated in laboratory animals. In rabbits, Murray *et al.* (3) suspected the liver to be the site of degradation of the hemoglobin-haptoglobin complex. No "free" hemoglobin appeared in the urine even when the injected hemoglobin exceeded the haptoglobin level. However, this is at variance with the findings of Franklin and his associates(4) who were unable to demonstrate either reticulo-endothelial participation or preferential hepatic removal of

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TABLE I. Blood Radioactivity Following Intravenous Injection of Different Doses of I^{131}-Labeled Hemoglobin. Each group contains 8 rats.

Dose in mg/100g body wt	Time in min after injection								
	5	10	15	30	60	90	120	180	240
3.0	87.6*	72.1	63.0	47.0	30.6	20.9	16.7	12.3	10.7
	±2.2†	± 2.3	± 3.5	± 3.8	± 3.8	± 3.6	± 2.7	± 1.4	± 1.1
5.5	87.2	69.0	57.3	43.4	28.6	23.2	21.2	14.3	11.76
	± 3.1	± 2.2	± 2.4	± 1.4	± 1.4	± 2.3	± 3.5	± 1.24	± .8
7.8	85.2	68.3	57.1	41.9	29.6	23.6	19.6	14.5	14.3
	± 2.6	± 1.6	± 1.9	± 1.2	± 1.8	± 2.5	± 1.8	± 1.6	± 1.6
10.2	85.7	73.1	63.9	44.9	32.5	24.2	20.4	15.0	13.1
	± 4.0	± 2.5	± 2.4	± 1.2	± 2.7	± 2.4	± 2.4	± 1.5	± 1.3

* The values are expressed as % of 2-minute value.
† Standard error of mean.

the hemoglobin-haptoglobin complex in the rat. Since these reported differences in plasma hemoglobin clearance mechanism may reflect species variations, the present study was undertaken further to investigate the fate of intravenously injected hemoglobin in the rat.

Materials and methods. Heparinized rat blood was collected by cardiac puncture. After removal of the plasma, the erythrocytes were washed twice with ice cold 0.85% NaCl. Hemolysis was achieved by addition of twice the erythrocyte volume of distilled water and $NaHCO_3$ (in substance) to achieve a final concentration of 1.5 g per 100 ml of fluid(5). After apparent completion of hemolysis, the solution was centrifuged to remove particulate matter and the supernatant was used for tagging.

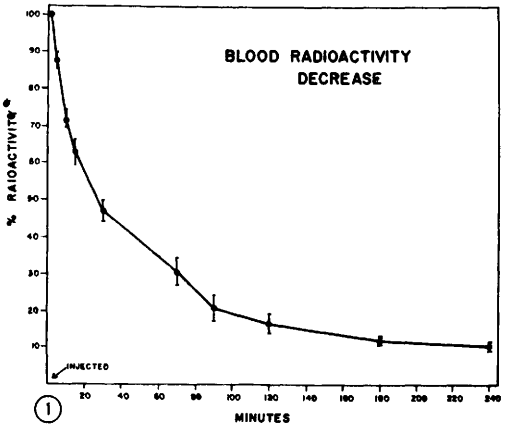
Labeling was achieved by treating the hemoglobin solution with Na I^{131} in the presence of an oxidizing compound ($KClO_4$) to liberate I_2^{131}; the unbound radioactivity was removed by exhaustive dialysis. In the final preparation at least 95% of the radioactivity was hemoglobin-bound, with less than 2 atoms of I^{131} per hemoglobin molecule. The site of the I^{131}-tag was determined after trypsin digestion of the labeled hemoglobin. The digestate was fractionated by using a Sephadex† column, and more than 90% of the radioactivity was found associated with tyrosine(6). Hemoglobin concentration was assayed by the cyanmethemoglobin method(7). Repeatedly during the course of the study, batches of labeled hemoglobin were checked for electrophoretic behavior, binding to serum haptoglobin, and peroxidase activity for possible detectable denaturation due to the labeling procedure.

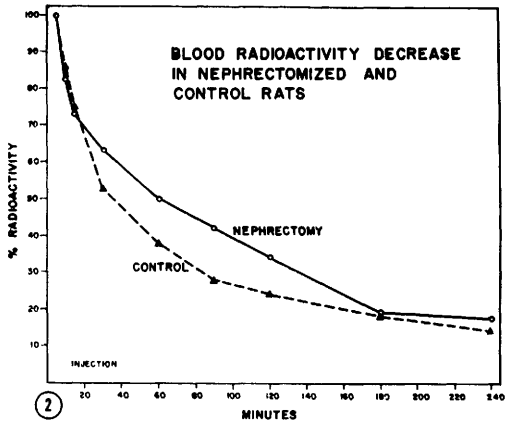
Young adult Wistar rats, weighing 150-250 g, bred in our laboratories, were used in all experiments. Under light ether anesthesia, graded amounts of I^{131}-labeled hemoglobin were injected into a saphenous vein. Subsequently, blood samples, 20-50 μl each, were collected periodically from the incised tip of the tail. Each sample was then added to one ml of distilled water and the contained radioactivity was determined in a well-type scintillation counter.

Serum haptoglobin was determined according to the activation method of Jayle and confirmed by the paper electrophoresis technic closely following the methods reported by Nyman(8). In a group of 18 rats the serum haptoglobin content varied from 118 mg per 100 ml serum to apparently none; four rats' serum exhibited no demonstrable haptoglobin, and in only 2 rats did the level exceed 100 mg. The mean haptoglobin level was 46.7 mg per 100 ml serum with a standard error of mean of 9 mg.

Results. 1. *Clearance of blood radioactivity.* Eight rats were injected with 3 mg of tagged hemoglobin/100 g body weight and the blood radioactivity level followed. Fig. 1 shows the results. The decrease of blood radioactivity content was rapid; in 30 minutes half of the injected radioactivity had left the circulation. The initial clearance was an exponential function of time, but after an hour the rate gradually diminished.

† Pharmacia, Uppsala, Sweden.





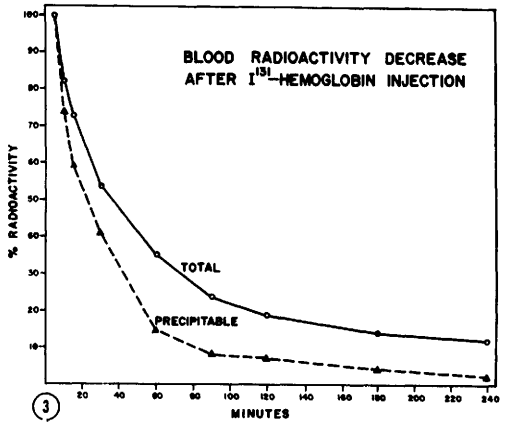


FIG. 1. Blood radioactivity level after i.v. inj. of I^{131}-labeled hemoglobin; 5 min. level is 100%. Each point represents 8 rats ± stand. dev.

FIG. 2. Blood radioactivity level after intrav. inj. of I^{131}-labeled hemoglobin; 6 rats; bilateral nephrectomy preceding inj.; 6 controls, "Sham" surgery, 5 min. blood level is 100%.

FIG. 3. Blood radioactivity level in 6 rats after i.v. inj. of I^{131}-hemoglobin. Total blood radioactivity and Folin-Wu-precipitable radioactivity determined on same samples.

2. Relation of amount of hemoglobin administered to its clearance rate. To test the capacity of the clearing mechanism, 4 groups of 8 rats each were given increments of I^{131}-labeled hemoglobin. Table I summarizes the findings in these experiments. Within the range tested, the clearance rate of circulating radioactive hemoglobin was apparently unaffected by administration of increasing amounts of hemoglobin.

3. Role of kidneys in clearance. To evaluate the possible renal contribution to the clearance of radioactivity from the blood, 6 rats were nephrectomized immediately prior to injection of 9.7 mg of radioactive hemoglobin per 100 g of body weight. A comparable group, sham-nephrectomized, served as controls. Fig. 2 shows the blood radioactivity in the nephrectomized animals. During the first 15 minutes after injection, blood radioactivity decreased at a rate identical to that in control animals. After 30 minutes, however, the radioactivity remaining in the blood was significantly higher in the nephrectomized group. In another group of 6 female rats a No. 22 polyethylene tubing was introduced into the bladder *via* the urethra and the urine continuously collected. Adequate urine flow was maintained by periodic administration, through a gastric tube, of 2% ethanol. When mild but constant diuresis had become established 3 mg per 100 g body weight of I^{131}-tagged hemoglobin was injected intravenously. Even the first urine sample, collected up to 5 minutes, contained some radioactivity. The urine remained clear and visibly free of hemoglobin, and the radioactivity could not be precipitated by the Folin-Wu reagent. Throughout the 3-hour observation period, urinary excretion rate of radioactivity remained rather constant. Average renal excretion per hour amounted to between 0.5 and 0.9% of the injected radioactivity in these rats.

4. Clearance of plasma hemoglobin. These results indicate that renal elimination plays an undetectable role during the initial phase of clearance, and that this pathway becomes significant after 30 minutes. It could be postulated that initially the blood radioactivity represents hemoglobin-bound I^{131} but later

the radioactivity represents breakdown products in addition to the bound forms. The kidneys may constitute a major route for clearance of blood radioactivity no longer bound to hemoglobin.

To test this theory, another group of rats was injected with 2 mg of I^{131}-tagged hemoglobin per 100 g body weight. Fifty microliter blood samples were collected at appropriate intervals and added to 1 ml non-radioactive hemoglobin (25 mg Hb/ml). After precipitation with a Folin-Wu reagent the radioactivity of the supernatant and precipitate was determined. The results are shown in Fig. 3. The rate at which hemoglobin-bound I^{131}, *i.e.*, precipitable radioactivity, leaves the plasma is an exponential function of time; it has a vascular half-life of 25 minutes. A slower exponential rate was noted after 80 minutes, when more than 90% of the injected radioactivity had already been removed. During the later part of the clearance the non-precipitable radioactivity represented a rapidly increasing portion of total plasma radioactivity.

Discussion. At the beginning of our plasma hemoglobin clearance kinetics studies presented here, we injected freshly prepared "cold" hemoglobin and attempted to follow the plasma hemoglobin concentration in tail blood samples. We soon found out, however, that collection and preparation of unhemolyzed plasma samples from the tail, even from a larger vein, is a formidable task. Because of this technical difficulty, most of our later studies were done with tagged hemoglobin. These early studies with "cold" hemoglobin showed a rapid plasma clearance identical with that obtained later with I^{131}-tagged hemoglobin. Furthermore, we found that the circulating radioactivity remained entirely protein-bound, at least for the first 30-45 minutes. Therefore, we feel that the decrease of radioactivity truly represented the clearance of the circulating hemoglobin.

In the rat, hemoglobin was cleared from the blood at an exponential rate unaffected by increasing the amount injected from 3 mg to 10.2 mg per 100 g body weight. Assuming the blood volume of the rat to be 7.2 ml per 100 g body weight(9), of which 55% is plasma, the initial augmented plasma hemoglobin level must have been 75 and 260 mg per 100 ml of plasma respectively. These high concentrations of plasma hemoglobin by far exceeded the haptoglobin levels of the rats. Our haptoglobin determinations were in the same range as reported by Ivanyi(10), *i.e.*, from apparent absence up to 118 mg per 100 ml serum. The hemoglobin dose range was selected with these data in mind. Nevertheless, neither renal excretion of "free", *i.e.*, not haptoglobin-bound hemoglobin, nor any overloading of the plasma clearance mechanism could be detected. Therefore, the significance of haptoglobin, so important in the human, is not immediately apparent in the rat.

The circulating hemoglobin is cleared rapidly in the rat, but this is without renal excretion. The radioactivity which soon after injection of the tagged hemoglobin appeared in the urine and was excreted at a rather constant rate, represented I^{131} which apparently was unattached to hemoglobin, since it could not be precipitated with the Folin-Wu reagent. This finding is directly at variance with some previous observations(2). This dissimilarity of results could be due to species variation as well as to the use of different labels in these studies. When Fe^{59} is used for metabolic follow-up of hemoglobin, a very rapid entrance of this tag into the iron pathways may distort and blur the actual catabolism of hemoglobin. In a few pilot experiments we found the clearance rate of Fe^{59} labeled hemoglobin to coincide initially with that of I^{131}-tagged hemoglobin. The plasma radioactivity curves became dissimilar only after 20-30 minutes; however, this is due to differences in the metabolic pathways of the radioactive tags used, rather than to other causes. In case of Cr^{51} tagging of hemoglobin an additional complication must be considered. When Cutler(11) used a Cr^{51} tag for his red cell clearance studies, he assumed that all the Cr^{51} within the erythrocytes was attached to hemoglobin. This common but erroneous assumption invalidates some of Cutler's data. In our laboratory the tagging process using Cr^{51} was extensively investigated(12). When Cr^{51}-la-

beled red cells were hemolyzed and passed through an appropriate Sephadex column (13) two components were obtained. One radioactive peak was associated with hemoglobin whereas the subsequent peak represented Cr^{51} not attached to hemoglobin. The relative amount of unbound chromium was variable in different species even though the tagging conditions were identical. In the rat we found approximately half of the chromium not bound to hemoglobin, whereas in the dog and especially in the human, the proportion of unattached hemoglobin was considerably smaller. When Cr^{51}-tagged hemolysate was injected into the rats a very fast plasma clearance and renal excretion of non-hemoglobin-bound Cr^{51} was observed. This was distinctly different from the clearance rate and catabolic fate of hemoglobin-bound Cr^{51}(12).

In the light of these findings, we believe that the catabolic studies with radioactive hemoglobin require very careful interpretation. Perhaps some of the existing discrepancies between human studies(14) and animal experiments could be resolved on the basis of species differences. Other apparent dissimilarities might be due to artifacts caused by the use of different radioisotope tags.

Summary. 1. The plasma clearance of I^{131}-tagged, autologous hemoglobin was studied in the rat. 2. This radioactive hemoglobin was cleared at an exponential rate, having a half-life of 25 minutes. 3. Within the range of 3 to 10.2 mg hemoglobin per 100 g body weight, clearance rate was independent of dose administered. 4. No renal elimination of hemoglobin could be demonstrated. 5. Rapid release of I^{131} from the hemoglobin was noted. This considerably increased the plasma radioactivity value over the actual hemoglobin level after only 30 minutes.

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A Toxin in the Coelomic Fluid of Scalded Starfish (*Asterias forbesi*).* (27337)

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The toxic-factor theory of the lethal effects of burns states simply the burned tissue releases a toxin which upon entering the circulatory system may induce shock and/or death. The work of Heilbrunn *et al.*(1) and Prinzmetal(2) suggested that toxins are present in a variety of vertebrate organisms. Rosenthal(3) found such toxins in the skin of rats. Simonart(4,5) reported that death, usually preceded by shock following severe scalding, is in part due to a toxic substance released at the site of injury. He found that

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