Sw. and of *Turnera ulmifolia* L. had a distinct protective effect on the development of dietary hepatic injury and of the accompanying acute necrotizing nephrosis, in rats fed a cirrhosis producing diet. The protective factor appears to be different from choline, methionine and Vit. B_{12}. It is not a protein or lipid.

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Kinetics of Plasma Hemoglobin Catabolism in the Rat. II. Cr^{51}-Tagged Hemoglobin.* (28321)

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Previous studies in this laboratory using I^{131}-tagged hemoglobin(1) revealed that the rat can clear and catabolize large amounts of intravenously injected hemoglobin with no indication of saturation of the plasma hemoglobin clearing mechanism. However, when Cr^{51} was used as a tag in place of I^{131}, marked discrepancies were noted. It soon became evident that Cr^{51}-labeling itself created specific conditions which altered the apparent plasma hemoglobin clearance. Furthermore, the metabolic fate of the Cr^{51} differed from that of I^{131}. This study was made to elucidate some peculiarities of Cr^{51} as a tag for hemoglobin.

Materials and methods. Fresh rat blood, collected by cardiac puncture, was added to half a volume of ACD† and approximately 30 μc $Na_2Cr^{51}O_4$‡ for each ml of blood. After this mixture was incubated for 45 minutes at 37°C, it was centrifuged, the supernatant was removed and the red cells washed twice with cold physiological saline. Then the red cells were lyzed with twice the volume of distilled water. Sodium bicarbonate was added, in substance, to give a final concentration of 1.5 g per 100 ml of fluid(1). The hemolysate was centrifuged for 25 minutes at 4500 rpm to remove all particulate matter. All hemoglobin solutions were prepared on the day they were used. For fractionation, the lysate of Cr^{51}-labeled rat erythrocytes was passed through a Sephadex§ column.

The Sephadex column was prepared in the following manner: "grade G-25 medium" Sephadex was suspended in physiological saline and the fine particles were removed by decantation before packing the column; elution was achieved with saline. For preparative fractionation, a column about 32 cm high and 2.5 cm in diameter was used; for qualitative studies, a smaller column (10 to 15 × 0.6 cm) was found practical.

For injection, the hemolysates were diluted to a hemoglobin concentration of 4 to

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† Trisodium citrate: 1.32 g, citric acid: 0.48 g, Dextrose: 1.47 g per 100 ml water.

‡ Rachrcmate, Abbott Laboratories, North Chicago, Ill. The specific activity of the different preparations varied from 130 to 400 mc Cr^{51}/mg Cr. This represents an average of 0.5 μg chromium per ml blood.

§ Pharmacia Fine Chemicals, Rochester, Minn.

7 mg/ml as determined by the cyanmethemoglobin method(2). Usually, 0.6 ml of the dilute hemolysate was injected into the saphenous vein of young adult Wistar strain rats. Blood samples (50 to 200 μl) were taken at specific intervals from the incised tail. Blood radioactivity content was expressed as per cent of injected Cr^{51}(3).

Urine samples were collected *via* a No. 20 polyethylene catheter inserted through the urethra of female rats, as previously described(1). These animals first received a standard dose of tranquilizer‖ intraperitoneally. One hour later, they were lightly anesthetized with ether, immobilized on their backs, and a No. 20 polyethylene tubing was introduced into the stomach *via* the esophagus. Through this tube, 5 to 10 cc of 2% ethyl alcohol in 0.2% sodium chloride was administered, slowly and continuously. Then, 12% ethyl alcohol (in 0.2% sodium chloride) was given, as needed, to assure a urinary excretion between 0.2 to 0.3 ml per 10 minutes. When a constant, satisfactory excretion rate was established, the radioactive solution was injected.

Organ distribution of Cr^{51} was studied by determining the amount of radioactivity in the different tissues. Radioactive materials were injected intravenously into groups of ether-anesthetized rats and, at the appropriate interval, blood was withdrawn prior to sacrificing the animals. After the skin was removed, the carcass was digested with sulfuric acid. The amount of radioactivity in each organ and the carcass was calculated as a per cent of the total injection.

Radioactivity of all samples was measured in a well-type scintillation counter, below 5% accuracy.

Results. 1. *Cr^{51}-tag in the hemolysate.* In the first phase of our studies, lysates of Cr^{51}-tagged rat erythrocytes were injected intravenously and the blood radioactivity level and renal excretion of Cr^{51} were followed. There were 2 rates of blood radioactivity clearance: an initial rapid decrease

‖ 1 mg/100 g body weight of Trilafon (hydroxyethyl chlorophenothiazinyl-propyl-piperaine), kindly supplied by Schering Corp., Bloomfield, N. J.

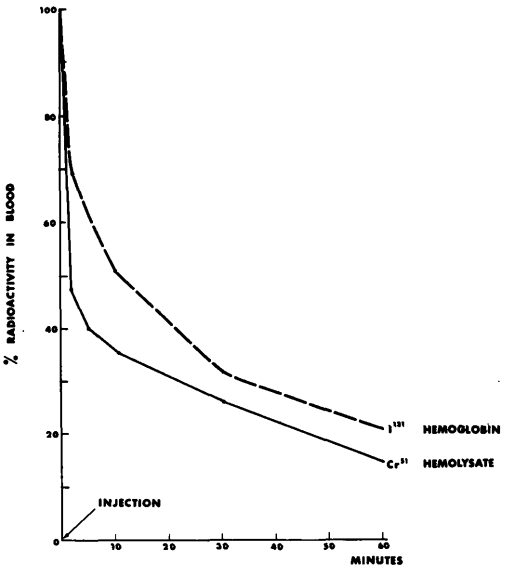


FIG. 1. Blood radioactivity after intravenous injection of homologous I^{131}-tagged hemoglobin and lysate of sodium radiochromate labeled homologous erythrocytes. Each point represents 15 rats. Note significant differences during first 10 min.

(Fig. 1), followed by a second, slower component. The slow rate corresponded to the fall off observed when I^{131}-labeled hemoglobin was injected(1). However, the initial fast blood clearance rate was associated with rapid appearance of Cr^{51} in the urine. No hemoglobin, but much radioactivity was contained in the urine samples collected 10 to 20 minutes after injection. These observations suggested that in the lysate of tagged cells, some of the Cr^{51} might not have been bound to hemoglobin.

To investigate this possibility further, the hemolysate was passed through a small Sephadex column. Drop-by-drop collection of the effluent fluid was started when the sharp red hemoglobin zone approached the bottom of the column. The radioactivity of each drop was determined and plotted against drop number. Fig. 2 A shows a typical analysis. The first peak (Fraction I) of radioactivity paralleled the increase and decrease of the hemoglobin content of the effluent fluid. This was followed by a second peak of radioactivity (Fraction II) without associated hemoglobin. There was no radioactivity in the subsequent drops and full re-

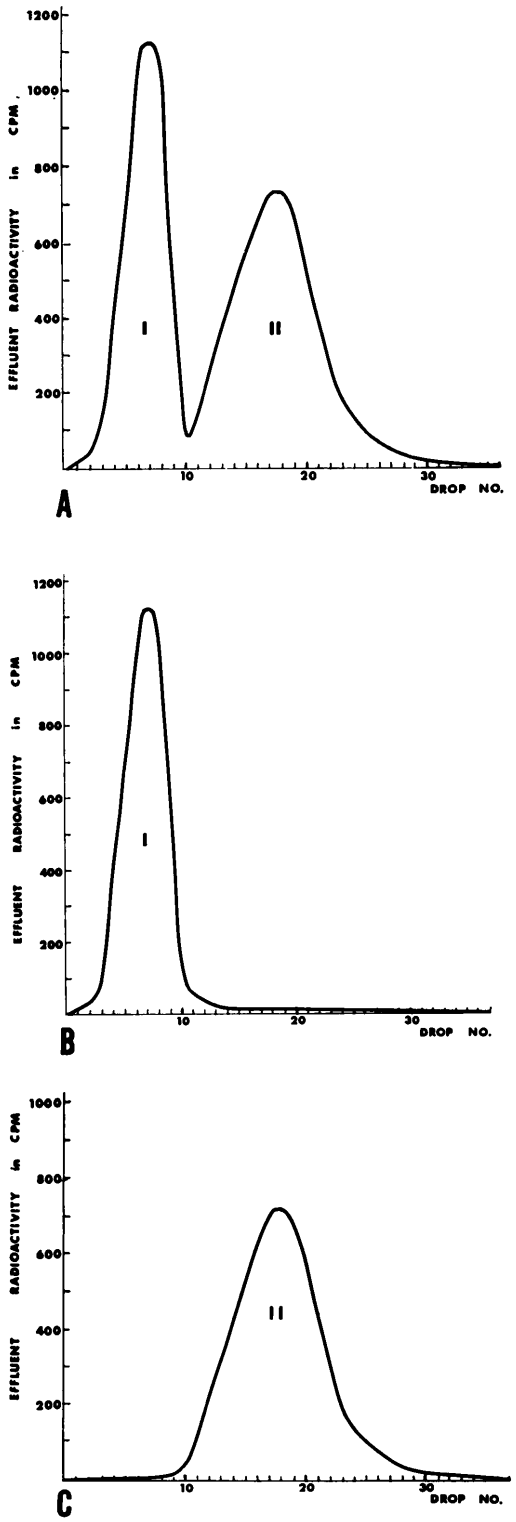


FIG. 2. (A) Radioactivity in effluent when lysate of Cr^{51}-tagged erythrocytes is passed through a Sephadex column. (B) A sample of Fraction I, and (C) a sample of Fraction II passed through a second Sephadex column. Note positions of single peak in each.

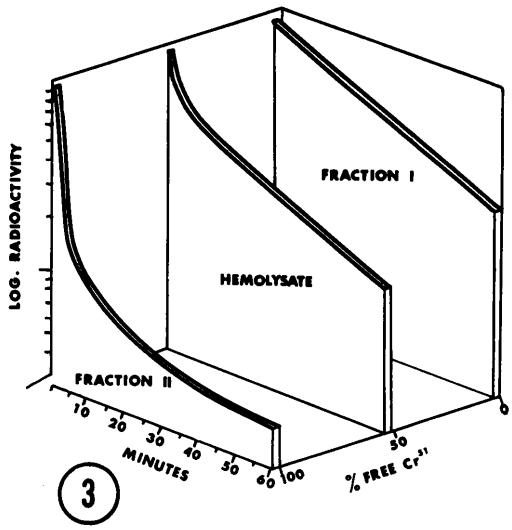
covery of the applied radioactivity was realized. When a sample from Fraction I was passed through another Sephadex column, only one peak of radioactivity still associated with hemoglobin concentration was found (Fig. 2B). Conversely, when a sample of Fraction II was first mixed with untagged hemoglobin to serve as an indicator, the mixture separated completely in the Sephadex column and the hemoglobin fraction was followed by a single radioactive peak (*cf* Fig. 2 C). The relative distribution of Cr^{51} between hemoglobin-bound and unbound fractions was determined in 20 different rat erythrocyte lysates prepared for the different experiments. Hemoglobin-bound Cr^{51} amounted to 46.6% (S.D. 12.96) and unbound Cr^{51} totaled 55.4% (S.D. 12.52).

In additional studies, the hemolysate was treated with Folin-Wu reagent. The precipitated radioactivity equalled the amount appearing as hemoglobin-bound Fraction I, as described above. When a mixture of original $Na_2Cr^{51}O_4$ solution and Fraction II was subjected to descending paper chromatography, the two components appeared as separated fractions.

Fraction II was retained when passed through an anion exchange resin, as was the commercial $Na_2Cr^{51}O_4$ solution.

In further studies, we tried to label erythrocytes with Fraction II using the standard procedure, as described above. The plasma and the washing fluids contained nearly all of the radioactivity. Although a small amount (in 2 separate experiments, 1.8 and 1.2%) of the added radioactivity entered the red cells during the incubation, Sephadex fractionation after lysis of these erythrocytes showed that the intra-erythrocytic Cr^{51} was not bound to hemoglobin.

2. *Blood clearance kinetics studies.* A standard dose of Cr^{51}-tagged erythrocyte lysate (containing both hemoglobin-bound and unbound Cr^{51}) was injected intravenously



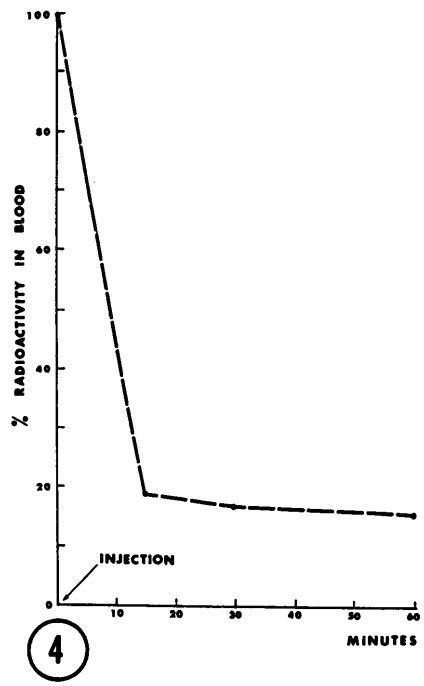


FIG. 3. Blood radioactivity following intravenous injection of (a) pure first (*cf.* Fig. 2), (b) pure second fraction and (c) unfractionated lysate of Cr^{51}-tagged rat erythrocytes; 12 rats in each group.

FIG. 4. Blood radioactivity after intravenous injection of $Na_2Cr^{51}O_4$. Each point represents 48 rats.

into a group of 12 rats and the blood radioactivity followed. Another equal-sized group was injected with pure Fraction I, in which practically all radioactivity was hemoglobin-bound. A third group of 12 rats was injected with Fraction II, *i.e.*, the second component of Sephadex fractionation containing no hemoglobin. Fig. 3 shows the results. The precipitous drop of radioactivity following injection of Fraction II in the blood is apparent. However, when all Cr^{51} injected was hemoglobin-bound, a single rate exponential clearance was noted. The blood Cr^{51} curve of the hemolysate clearly shows the contribution of both components. Since the original hemolysate contained both hemoglobin-bound and unbound Cr^{51}, the beginning of the curve is dominated by the clearance of Fraction II. For further comparison, $Na_2Cr^{51}O_4$ solution was given to 48 rats. These results are shown in Fig. 4.

3. *Urinary excretion of Cr^{51}.* The excretion patterns gave further insight into the metabolic fate of Cr^{51} (Fig. 5). Fraction II was cleared rapidly from the circulation and was recovered in the urine. More than half of the Cr^{51} was present in the urine 30 minutes after the injection, and at the end of the 3 hours, about 78% had been excreted. The excretion pattern differed when Fraction I was injected. Only 8.41% was excreted during the first hour, and 12.8% by the end of 3 hours. Comparing the urinary findings with the corresponding plasma-clearance data, it is evident that (a) the hemoglobin-bound Cr^{51} was removed from the blood in an exponential fashion and (b) after initial rapid urinary excretion amounting to 8 to 9%, the rest of Cr^{51} was excreted slowly. In contrast, the non-hemoglobin bound Fraction II was (a) cleared from the plasma very rapidly, and (b) excreted by the kidney efficiently.

To clarify this point further, injections were made with the original lysate of Cr^{51}-tagged erythrocytes. Since this contains both Fraction I and II, the urinary excretion curve took an intermediate position, as expected (Fig. 5), reflecting an initial, accelerated blood clearance with corresponding urinary excretion; the later part of the blood radioactivity curve, as well as the renal excretion pattern, approached that of Fraction

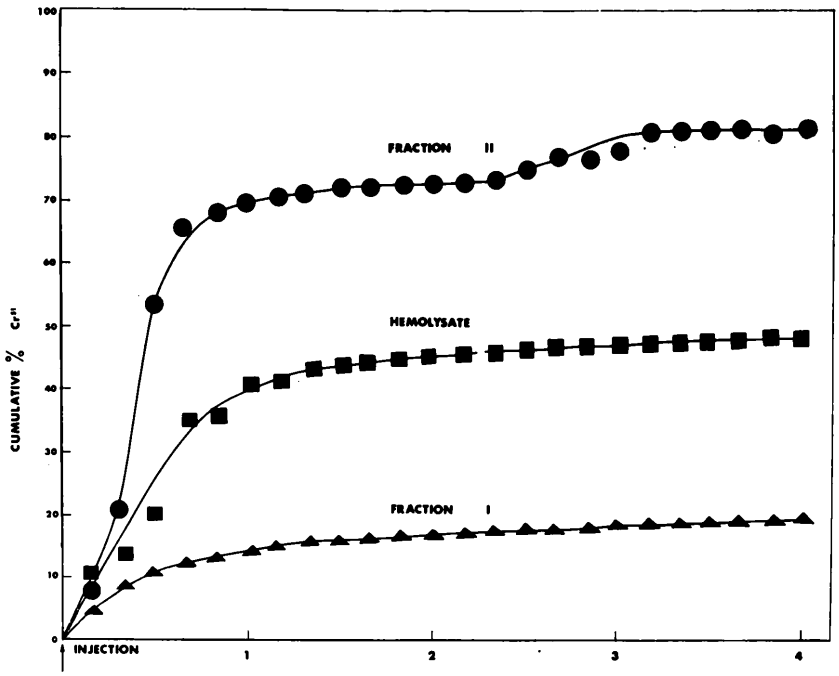


FIG. 5. Urinary excretion of Cr^{51} after intravenous injection of the same preparations as in Fig. 3.

I, since most of Fraction II had been excreted. The urinary radioactivity was, in no instance, associated with hemoglobin.

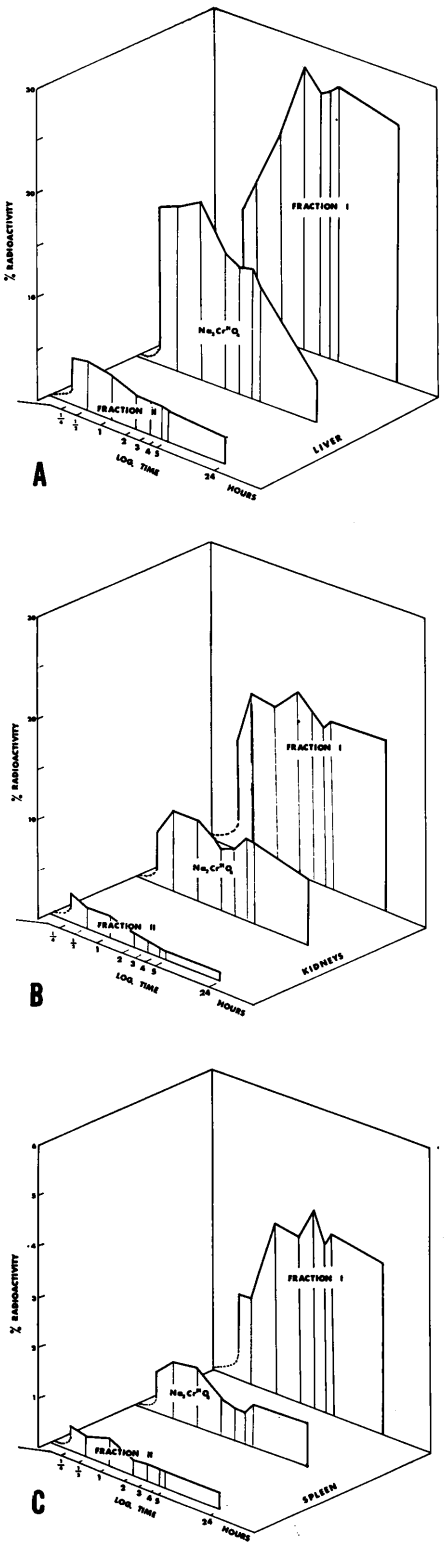
4. *Tissue distribution of Cr^{51}.* Three groups of rats, 48 animals each, were studied. Group A was injected with Fraction I, *i.e.*, the purified hemoglobin-Cr^{51} preparation; Group B with Fraction II; and Group C with the diluted commercial $Na_2Cr^{51}O_4$. At intervals, rats of each group were sacrificed and the radioactivity content of the different tissues determined. Fig. 6 a, b, and c show some of these data. When the pure Fraction I was injected, the hepatic radioactivity steadily increased during the first 2 hours, remained the same for several hours, and then slowly decreased. A day later, most of this radioactivity was still there. The spleen removed more hemoglobin per gram tissue than the liver. Kidneys contained much Cr^{51} 30 minutes after the injection, which remained unchanged throughout the 24-hour observation period. The radioactivity content of the carcass decreased, slowly and steadily, from around 30% at 15 minutes, to 16% at 24 hours.

When Fraction II was injected, the tissue distribution pattern was different (*cf* Fig. 6 a, b, and c). Only a small portion of the radioactivity was found in the liver, spleen, and kidneys throughout the 24-hour period.

Following the intravenous injection of sodium radiochromate, an immediate hepatic uptake of 15% was noted, whereas the splenic localization was much lower (*cf* Fig. 6 c). For the first few hours after injection, 35% injected Cr^{51} was present in the carcass, which decreased slowly.

5. *Repeated injection of hemoglobin-Cr^{51}.* A group of rats was injected with Fraction I and urinary exretion of Cr^{51} was followed. Three hours later, when the Cr^{51} excretion had diminished, another equal dose of Fraction I was injected. This resulted in a second wave of radioactivity excretion identical to the initial pattern.

6. *Canine hemoglobin.* A group of 10 rats was injected with a preparation of dog hemoglobin-Cr^{51} from which the non-hemoglobin-bound Cr^{51} (Fraction II) had been removed. This canine Fraction I was cleared from the circulation of the rat essentially



the same way as homologous Fraction I. Also, the urinary excretion pattern of Cr^{51} was the same with both rat and canine hemoglobin-Cr^{51}.

Discussion. I^{131} as a hemoglobin label was successfully used in our laboratory to follow the plasma clearance and subsequent catabolism of autologous hemoglobin in the rat(1). Since Cr^{51}-tagging of erythrocytes is a simple procedure, it appeared to us at the start of our present work that Cr^{51} and I^{131}-tagging could be used interchangeably in hemoglobin metabolic studies. Pilot experiments revealed clear differences between I^{131}-labeled hemoglobin and Cr^{51}-tagged hemolysate. The discrepancy called for closer study of the nature of the Cr^{51}-tag once within the erythrocyte membrane. This seemed important since Cr^{51}-tagged erythrocytes are widely used in clinical and investigative hematology.

The consensus is that when red cells are incubated with radioactive sodium chromate, all the intraerythrocytic Cr^{51} is firmly attached to hemoglobin(4,5). Gray and Sterling(6) reported that 97% of the radioactivity is hemoglobin-bound and 2% is in the washed stroma. Yet our data, reported here, clearly show that this is incorrect. Within the red cells, only a variable portion of Cr^{51} is hemoglobin-bound. After hemolysis, the Cr^{51} fraction not bound to hemoglobin (Fraction II) could be readily separated (a) by precipitating the hemoglobin, and (b) by passing the hemolysate through a Sephadex column. This Fraction II was also easy to distinguish from sodium chromate (a) by paper chromatography (but not by anion exchange resin which absorbed both), and (b) by the inability of Fraction II to label erythrocytes. Interestingly, Bigby *et al.*(7) reported that 96 hours after radioactive sodium chromate was injected intraperitoneally into golden hamsters, the peritoneal washing lost its "capacity for tagging" normal erythrocytes. This may represent a conversion of chromate into Fraction II.

In vitro, Fraction II could readily be sepa-

FIG. 6. Hepatic, renal, splenic radioactivity after injection of (A) Fraction I, (B) Fraction II (*cf.* Fig. 2), and (C) $Na_2Cr^{51}O_4$; 48 rats in each group.

rated from the bound Fraction I. This separation occurred also *in vivo*. When the hemolysate was injected intravenously, the rat cleared the 2 fractions from the circulation in a very different way. The hemoglobin-bound Fraction I was removed from the plasma like hemoglobin-I^{131}, presumably by the reticuloendothelial system. Fraction II, however, left the body promptly *via* the kidneys, seemingly without ever entering the host's metabolism. Thus the metabolic inertness of Fraction II was reflected *in vitro* by its inability to tag erythrocytes, and *in vivo*, by its prompt urinary excretion. Then too, urinary Cr^{51}, collected after intravenous injection of pure Fraction I, also lacked the ability to tag erythrocytes. It is possible that not only the erythrocytes *in vitro*, but also the tissue cells which catabolize hemoglobin-Cr^{51}, convert the tag into its metabolically inert form. Studies by Schenk(9) indicate further that when the circulating Cr^{51}-tagged erythrocytes undergo sudden hemolysis, induced by acetyl phenylhydrazine, the released Cr^{51} is not reutilized. Perhaps the elution of Cr^{51} from the intact erythrocytes *in vitro*(8) and *in vivo*, represents our Fraction II. But further studies are needed to prove this.

These data could be explained by the following hypothesis: During the catabolism of the Cr^{51}-tagged erythrocytes, as well as during the breakdown of the Cr^{51}-labeled hemoglobin, the radioactive tag is converted into its metabolically inert form prior to its excretion. Presumably, conversion takes place within the reticuloendothelial cells which removed the hemoglobin molecule.

The routine clinical labeling procedure calls for addition of ascorbate at the end of incubation to reduce the extraerythrocytic Cr^{51}. In the experiments reported here this step was omitted. Instead, the entire plasma was quantitatively removed after incubation and the red cells thoroughly washed before hemolysis. This resulted in removal of all extraerythrocytic Cr^{51}. In similar studies with human blood, it was noted that addition of ascorbate played no role in the intraerythrocytic behavior of Cr^{51}.

In previous studies, when the catabolism of Fe^{59} or I^{131}-tagged hemoglobin was traced, a considerable amount of the liberated tag appeared in the general metabolism a few minutes after injection(1). By contrast, when Cr^{51} was liberated, some was promptly excreted. Yet after the initial rapid excretion of 6 to 8% of the Cr^{51}, subsequent excretion was very slow. Although the Cr^{51} binding by hemoglobin is still obscure(10,11), one might speculate that the excretion pattern indicates different types of bindings of Cr^{51} on the hemoglobin molecule. One type is readily split and the Cr^{51} converted to its inert form; this binding accounts for 6 to 8% of all the hemoglobin-attached Cr^{51}. To test this speculation, another dose of Fraction I was given 3 hours after the initial injection, when urinary Cr^{51} output had all but ceased. An excretion pattern identical with the first was obtained, apparently by supplying the reticuloendothelial cells with more readily splitable Cr^{51} substrate. When canine hemoglobin-Cr^{51} was given to rats. the liberation and excretion pattern of Cr^{51} was the same as following autologous hemoglobin injection, suggesting that species inherent metabolic traits cause this variation in Cr^{51} binding, rather than possible species differences of the hemoglobin molecule itself. This was confirmed by our observations in the dog(12). Here, both rat and dog hemoglobin-Cr^{51} was cleared from the circulation in the same way, and the urinary excretion pattern was similar to that noted after I^{131}-labeling: namely, a constant-rate clearance. Pilot studies in humans indicated that some of the Cr^{51}-tag was rapidly cleared in the urine, but most of it was released slowly from the liver, spleen, and other reticuloendothelial cell-containing tissues. In these cases, body surface counting and urine studies agreed with the findings of Jandl *et al.* (13), suggesting similarity with the rat.

The relative amount of Fraction II within the erythrocytes is apparently species dependent. In the rat, less than half of the intraerythrocytic radioactivity was hemoglobin bound whereas in the dog, Fraction II was only a few per cent. Our preliminary

studies in humans indicate that 8 to 10% of Cr^{51} is Fraction II.

Most of the Cr^{51} is retained in the tissue where sequestration of erythrocytes occurs, so that by external counting it is possible to demonstrate in patients the site of erythrocyte sequestration. This is a routine procedure in clinical hematology. However, if hemolysis occurs within the blood vessels, the liberated hemoglobin will be cleared by the reticulo-endothelial elements of the liver, spleen, etc. Hence, external scanning will show a radioactivity distribution pattern as described here (Fig. 6 a, b, c). When following tagged red cell injection, marked accumulation of Cr^{51} in the spleen is demonstrable by means of external counting, splenectomy may be beneficial(14). Our studies seem to shed light upon the underlying mechanism responsible for the high splenic radioactivity in the case of selective splenic sequestration of erythrocytes. In contrast, intravascular hemolysis will result in hepatic and splenic deposition of Cr^{51} in proportion indicated on Fig. 6.

Summary. (1) Following *in vitro* labeling of erythrocytes with $Na_2Cr^{51}O_4$, a considerable amount of the Cr^{51} was found unattached to hemoglobin. (2) This unattached Cr^{51} lost the ability to label erythrocytes, and when given intravenously, was excreted rapidly by the kidneys. (3) Hemoglobin-bound Cr^{51} was cleared from the blood the same as was previously reported with I^{131}-hemoglobin. After clearance, however, much tissue retention of Cr^{51} was noted. (4) Rats metabolized homologous and canine hemoglobin similarly, with slow excretion of Cr^{51} by the kidneys. (5) The clinical implications of these findings indicating some peculiar metabolic characteristic of Cr^{51}-tagged erythrocytes are discussed.

The authors wish to express their appreciation for the assistance of R. Dufort, B. Stoesser, J. Goldstein and R. Rohe.

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Vasopressin Antagonism by Azacyclonol in Hydrated Rats.* (28322)

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An antidiuretic action of azacyclonol (α-4-piperidyl diphenyl carbinol hydrochloride) in the rat has been reported by Sanchez and Davis(1). Release of antidiuretic hormone (ADH) by the drug was investigated and excluded as a possible mechanism of this ac-

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