Hemolysis and Erythropoiesis
V. The Utilization of Hemoglobin Iron by Bled Anemic Dogs*
(33858)

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(Introduced by A. S. Gordon)
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Our previous observations have shown that intraperitoneal administration of autohemolysates accelerates the recovery from anemia in bled dogs (1). They were performed in 4 dogs which were bled on two occasions to obtain similar degrees of anemia. Erythrocytic production was measured in the 14- to 18-day lapse following each bleeding. Each animal's production during the control observation was compared with that attained in the same dog when the hemolysate was given. The gain in total circulating hemoglobin was 60.7% greater under hemolysate administration. We suggested that this was due to a stimulatory action of the hemolysate (1). It has been considered, however, that the response observed in this and other experiments to hemolysate administration may be due to increased availability of hemoglobin building materials, particularly iron (2–4). The present paper reports the results of an experiment aimed to elucidate whether hemoglobin iron was responsible for the increased erythrocytic production reported previously (1). The methodology was the same as in the original paper (1), except for the use of a hemolysate containing hemoglobin-^{59}Fe in the present study.

Material and Methods. EDTA was used as anticoagulant for all blood samples. Hemoglobin was estimated by the cyanmethemoglobin method. The packed cell volume was determined in Wintrobe tubes centrifuged at 2200*g* for 30 min.

Sodium chromate labeled with ^{51}Cr with a specific activity of 50 μCi/μg and ferric citrate labeled with ^{59}Fe with specific activity of 30 μCi/μg were the isotopes used. Radioactivity was measured in a well scintillation counter for a minimum of 15 min or 10,000 counts. When necessary, the two isotopes were differentiated by gamma spectrometry.

Two normal adult male dogs weighing 17 (dog A) and 14 kg (dog B), kept in individual cages, were rendered anemic by four 15–20 ml/kg bleedings on alternate days. On the day following the fourth bleeding, their erythrocyte volume was measured by the ^{51}Cr method (5). Immediately afterwards they received: (a) 100 mg of intramuscular iron dextran[1] daily until the amount of iron (450 mg) removed through the bleedings was replaced, and (b) daily intraperitoneal injections for 15 days of 1.5 ml/kg of a dog hemolysate labeled with ^{59}Fe. Two days after the last injection of hemolysate, the erythrocytic volume was measured again with the ^{51}Cr method. The amount of hemoglobin gained, and of total iron and of iron from labeled hemoglobin utilized in the synthesis of new heme pigment during the experimental period were determined in each animal. Total circulating hemoglobin (T Hb) was estimated by the following formula:

$$\text{T Hb} = \frac{\text{EV} \times \text{g of Hb in 100 ml of blood}}{\text{Hematocrit} \times 0.96},$$

where EV = erythrocytic volume in ml; factor 0.96 = hematocrit correction for trapped plasma.

The ratio of 3.47 mg of iron/g of hemoglobin was used to calculate the amount of iron utilized.

The dog ^{59}Fe labeled hemolysate was prepared as follows: one normal male dog weighing 25 kg was made anemic (hemoglobin 8.8 g/100 ml) by means of three bleedings. Immediately after the third bleeding he

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[1] Iron–dextran as Imferon was kindly supplied by Farmacéuticos Lakeside.

TABLE I. The Effect of Heme Iron on Hemoglobin Regeneration in Bled Dogs.

	Dog:	A	B
Hemoglobin (g/100 ml)	I[a]	7.7	7.8
	F	12.5	14.1
Erythrocyte volume (ml)	I	295.3	241.0
	F	505.9	551.3
Hemoglobin increment (g)		70.3	98.0
Amount of Fe used for Hb increment (mg)		243.8	339.9
Amount of Fe injected as ^{59}Fe–Hb (mg)		199.1	164.0
Heme–^{59}Fe incorporated into RBC			
Absolute values (mg)		33.9	40.5
Relative values			
(a) % of doses given		17.0	24.7
(b) % of iron in Hb gained		13.9	11.9

[a] I = initial; F = final; time elapsed, 16 days.

was intravenously injected with 130 μCi of ^{59}Fe and from the next day on and for 7 days he daily received 100 mg of iron–dextran intramuscularly. On day 13 the dog was bled 500 ml and on the next day, another 400 ml of blood were removed, under aseptic conditions in plastic bags. The bags were centrifuged, supernatant plasma was removed and the concentrated red cells were transferred from the bags to a sterile flask and mixed. Isotonic saline and tetracycline sufficient to achieve concentrations of 15 g/100 ml of hemoglobin and 14 μg of tetracycline/ml were added. After complete homogenization, the red cell suspension was divided into aliquots. The aliquots were frozen and thawed six times to insure complete hemolysis and then were kept frozen until use.

Results. The pertinent information is given in Table I. During the experiment only 17.0 and 24.7% of the hemoglobin iron given was reutilized for hemoglobin synthesis by dogs A and B, respectively. Thus hemoglobin iron accounted only for 13.9 and 11.9% of all iron present in the hemoglobin gain.

Discussion. As stated above, the experimental conditions of the present study were entirely similar to those of our previous work (1), in which it was shown that the hemoglobin gained by dogs with acute anemia due to blood removal and injected daily with 1.5 ml of hemolysate/kg and iron–dextran, was 60.7% greater than that observed in the control periods of the same animals following injections of iron dextran and saline. During the control experiment hemoglobin production was 0.112–0.377 g/kg/day (mean 0.246).

The present results show that dogs under these experimental conditions reutilize some of the iron given as hemoglobin for *de novo* hemoglobin synthesis but in an amount insufficient to account for the previous finding. The hemolysate iron contributed only 11.9 and 13.9% of the hemoglobin iron gained during the study period, which account for only a third of the 60% overproduction mentioned above (1.607 multiplied by 0.129). These results are not in accord with the hypothesis that the increased production induced by the hemolysate is due mainly to the availability of large amounts of heme iron and therefore indirectly support our original interpretation. Experiments performed in other species (6–8) also agree with our concept that red cell constituents stimulate erythropoiesis.

Summary. Two dogs with anemia induced by bleeding were injected intramuscularly with iron dextran to replace the iron lost through the bleedings. They were given intraperitoneally 1.5 ml/kg/day of a hemolysate containing 15 g/100 ml of ^{59}Fe-labeled hemoglobin. Seventeen and 24.7% of the injected heme-^{59}Fe was reincorporated in the RBC, which accounted for only 13.9 and 11.9% of the iron present in the hemoglobin gained following injections of hemolysate. These results indicate that the 60.7% increase in hemoglobin production after hemolysate administration observed in previous similar experiments (1), cannot be ascribed to availability of large amounts of heme iron.

1. Sánchez-Medal, L., Labardini, J., and Loría, A., Blood **21**, 586 (1963).

2. Castel, W. B., "Year Book of Medicine" p. 291. Year Book Publ., Chicago,Illinois (1964).

3. Keene, W. R. and Jandl, J. H., Blood **26**, 705 (1965).

4. Grant, W. C. and Root, W. S., Physiol. Rev. **32**, 449 (1952).

5. Labardini, J., Herrera, F. M. and Sánchez-Medal, L., Am. J. Clin. Pathol. **50**, 542 (1968).
6. Sánchez-Medal, L., Rev. Invest. Clin. Mex. **14**, 199 (1962).
7. Salas, M., Smyth, J. F., Sánchez-Medal, L., De Rodríguez, E., and Labardini, J., Med. Exptl. **8**, 159 (1963).
8. Labardini, J., Sánchez-Medal, L., Arriaga, L., López, Delifa, and Smyth, J. F., J. Lab. Clin. Med. **72**, 419 (1968).

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Amino Acids, Including Asparagine and Glutamine, in Plasma and Urine of Normal Human Subjects (33859)

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A recent development in the technology of amino acid assay via ion-exchange column chromatography was the separation of asparagine and glutamine by employing lithium citrate buffer systems (1) instead of the sodium citrate buffer systems usually used for determination of the acidic and neutral amino acids (AN) (2). We recently reported (3) our evaluation and modifications of the use of lithium buffers at flow rates employed in the Beckman/Spinco model 120 amino acid analyzer. Our major changes of the original method of Benson *et al.* (1) were the correction of an inadvertent interchange of the elution positions of citrulline and α-aminoadipic acid, and an improvement in the resolution in the cystine-to-leucine region by lowering the pH of the second buffer of the new, two-stage, lithium buffer system from 4.15 to 3.82.

The present report presents comparative results of the application of both the new and previous techniques for measuring free amino acids in plasma and urine of normal subjects. Also included are results of the determination of total amino acids found after acid hydrolysis of protein-free extracts of plasma and urine. The measurements of free and total aspartic and glutamic acids, as well as their respective amides, provided an opportunity to evaluate the contributions of these acids, their amides, and other conjugates to the total hydrolyzable aspartic and glutamic acids in plasma and urine.

Materials and Methods. Collection of samples for analysis. Fasting (overnight) blood samples (10 ml) were taken by venipuncture at three 6-month intervals from two female and three male, healthy, mature volunteers (wt range, 53–77 kg; age range, 24–42 years). The blood was immediately mixed with dry sodium heparinate (10 units/ml of blood), cooled in an ice bath, and centrifuged at 1500 rpm for 10 min. The plasma was removed and recentrifuged, and a portion was deproteinized immediately. The remainder was stored at —20° for later direct determination of tryptophan and creatinine.

Concurrent with the last two blood samplings, we also collected urine from the volunteers. The collection period was from 6:30 to 10:30 a.m., during which time the overnight fast was continued. Only water *ad libitum* was allowed. At the midpoint of the urine collection time, the blood sample was withdrawn. These conditions were dictated by what was considered to be the most practicable in the clinical situation for studies in uremic patients (4, 5), since the samples from these normal subjects were to serve as bases for comparison. The urine was refrigerated immediately after voiding. Following completion of the collection, a portion was deproteinized and the remainder was stored at —20°.

Deproteinization. Protein-free filtrates of plasma or urine for analysis of amino acids were prepared by the picric acid method (6). Urine was deproteinized in anticipation that samples from uremic patients would require