

RED CELL STROMA AND HEMOGLOBIN METABOLISM IN ANEMIC DOGS

REGENERATION OF RED CELL PROTEINS LABELED WITH C¹⁴ LYSINE*

BY G. H. TISHKOFF, M.D., C. L. YULE, M.D., F. S. ROBSCHUIT-ROBBINS, PH.D.,
AND G. H. WHIPPLE, M.D.

(From the Department of Pathology, The University of Rochester School of Medicine and
Dentistry, Rochester, New York)

(Received for publication, August 1, 1955)

Previous studies on the life span of red blood cells (2), into which C¹⁴-labeled amino acids had been incorporated, were concerned with whole cells and no attempt was made to distinguish the activity of hemoglobin from other red cell constituents such as protein and lipides of the stroma. The present report is concerned with a comparison of the C¹⁴ activity of erythrocyte hemoglobin with that in stromal protein throughout the life cycle of the red blood cell in 5 dogs. *DL*-Lysine- ϵ -C¹⁴ was fed to 3 animals with anemia produced by repeated bleeding, to one with anemia and hypoproteinemia, and to one with anemia due to phenylhydrazine. During the period of *active regeneration from anemia*, red cell hemoglobin and stroma protein exhibit some degree of independence as far as C¹⁴ incorporation is concerned. After stabilization of the hematocrit findings at a normal level, however, the rates of decline of C¹⁴ activity for these 2 proteins are approximately equal. It is suggested on the basis of the data presented that the proteins and non-specific lipide fractions of erythrocyte stroma, as characterized by the method used to obtain them, are metabolically inert during the life span of the circulating red blood cell beyond the reticulo-cyte stage. The same appears to be true for hemoglobin.

Methods

The methods employed for the preparation of dog red cell stroma, stroma lipide, and stroma protein have been described elsewhere (7). Stroma protein comprises the residue of stroma after the lipide has been extracted. Stroma was isolated in duplicate or triplicate from 4 ml. of packed, washed, red cells and fractionated. Crystalline dog hemoglobin was isolated from 5 ml. of packed cells and lyophilized, as described by Sumner and Summers (5). Stroma lipide was dried in a tared flask and weighed directly. Stroma and plasma nitrogen values were determined by the semimicroKjeldahl procedure and converted to protein by the factor 6.25. Total hemoglobin was measured in 20 c. mm. of blood by absorption spectrometry following

* This work has been supported in part by funds from the Office of the Surgeon General. We are also indebted to Eli Lilly and Company and to the Ernest L. Woodward Fund for aid in conducting this work.

conversion to the pyridine hemochromogen (4). Residual stroma hemochromogen was also assayed by the same method.

C¹⁴ activity was determined with a dynamic condenser electrometer after conversion of the organic material to CO₂ by wet oxidation (1). Materials used in the C¹⁴ assay consisted of 200 mg. of crystalline hemoglobin, 1 ml. of plasma, and the stroma protein and lipide from 4 ml. of packed red blood cells (7). Since stroma protein activities approached the background counts toward the end of each experiment, 2 or 3 samples were combined in order to obtain measurable activities. Measurable C¹⁴ activity of stroma lipide was present only in the earlier phases of 2 experiments. Single radioactivity determinations were made.

EXPERIMENTAL OBSERVATIONS

Details relating to the production, severity, and duration of anemia in the 5 dogs used in this study are outlined in Table I.

TABLE I
Type and Degree of Anemia

Dog No.	Sex	Weight <i>kg</i>	Rbc hematocrit		Plasma protein		Period of anemia <i>days</i>	Type of anemia
			Initial <i>per cent</i>	Anemic <i>per cent</i>	Initial <i>gm./100 ml.</i>	Anemic <i>gm./100 ml.</i>		
51-22	Female	13.8	55.6	31.1	5.57	4.87	30	Bleeding
51-194	Female	9.1	55.0	17.3	5.86	6.72	60	Bleeding
47-36	Female	20.0	50.0	23.2	—	6.45	56	Bleeding
45-2	Male	17.6	48.5	23.3	—	4.0	71	Bleeding and hypo-proteinemia
46-9	Male	20.9	53.3	21.9	—	7.4	68	Phenylhydrazine

The first 3 animals listed were rendered anemic by repeated heavy bleeding while receiving a daily diet of salmon bread (250 to 350 gm.), klim (skimmed milk) (20 gm.), and 50 to 75 gm. of canned salmon. Dog 45-2 received an almost protein-free diet during the last 16 days of the 71 day depletion diet in order to induce *hypoproteinemia* in addition to the anemia. The diet consisted of a protein-free biscuit (8) (450 to 600 gm.) containing adequate vitamins and minerals exclusive of iron. During this time the plasma protein concentration fell from 5.0 to 4.0 gm. per 100 ml. The hemolytic anemia was produced in dog 46-9 by the subcutaneous administration of daily doses of phenylhydrazine. The initial dose of 50 mg. was increased to 150 mg. by the 33rd day; a total of 68 doses was given.

At the conclusion of the anemic period each dog was placed on a *regenerating diet*. Dogs 51-22 and 51-194 received 150 gm. of cooked pig liver daily added to the low protein diet containing sucrose, bone ash, salt mixture, and vitamin supplements (6). Dogs 47-36 and 45-2 were fed cooked pig liver (250 gm.), salmon bread (300 gm.), Klim (20 gm.), and lextron¹ plus iron (7.5 gm.) daily.

On the 2nd day of this dietary regimen C¹⁴-labeled lysine was fed with a portion of the diet. General details pertaining to the regeneration period, including amounts of

¹ Generously supplied by Eli Lilly and Company.

protein and labeled lysine fed are shown in Table II. Stroma protein values in the second to the last column (Table II) are comparable to those previously observed in anemic dogs (7). The lowest anemic level for stroma protein occurred with mild anemia of short duration (dog 51-22) and the highest level for stroma protein (dog 46-9) was observed with hemolytic anemia. Values had reverted to the normal range by the time the hematocrit percentage had returned to preanemic levels.

Figs. A-D show curves constructed from C^{14} activities per gram of red blood cell hemoglobin and stroma protein in 4 experiments. Determinations were made initially 2 days after feeding C^{14} -labeled lysine² to the anemic dogs and

TABLE II
Period of Regeneration of Red Cells

Dog No.	Regeneration diet		<i>dl</i> -lysine- α - C^{14} fed	Complete recovery from anemia	Duration of experiment after C^{14} feeding	Stroma protein	
	Daily	Protein intake per kg.				Anemic level	After recov- ery
	gm.	gm.		days	days	mg. per ml. of packed red cells	
51-22	Liver 150 plus basic diet	3.3	75 mg. 64 μ c.	28	193	11.0	9.0
51-194		4.9	70 mg. 87.5 μ c.	30	150	14.3	9.6
47-36	Liver 250 plus lex- tron and iron	5.4	60 mg. 55 μ c.	37	157	12.4	9.0
45-2		7.6	50 mg. 40 μ c.	45	152	12.6	8.1
46-9	Liver 200 plus sal- mon bread	4.5	30 mg. (<i>l</i> -lysine) 27 μ c.	20	149	26.3	8.8

subsequently at intervals extending well beyond the normal red cell life span. Detailed results from a typical experiment are shown in Table III. Since the experimental procedures and results for dogs 51-194 and 47-36 were essentially similar, curves derived from the latter are not included.

Figs. A, B, and C (anemia produced by bleeding) may be roughly divided into 4 phases. The first of these, a period of increasing C^{14} activity, represents incorporation of the isotope into the 2 major protein fractions of the red cell. In the 2 examples of uncomplicated hemorrhage anemia illustrated (Figs. A and B) the maximum stroma protein C^{14} activity is reached at 4 days and is somewhat higher than the hemoglobin activity which reaches a peak on the 6th day. In the third dog with anemia due to bleeding (47-36) (Table I) and

² The method of Olynyk (Olynyk, P., Camp, D. B., Griffith, A. M., Woislowski, S., and Helmkamp, R. W., *J. Org. Chem.*, 1948, 13, 405) was used to procure the labeled lysine. It was made in the chemical laboratories of the University of Rochester and those of its School of Medicine and Dentistry.

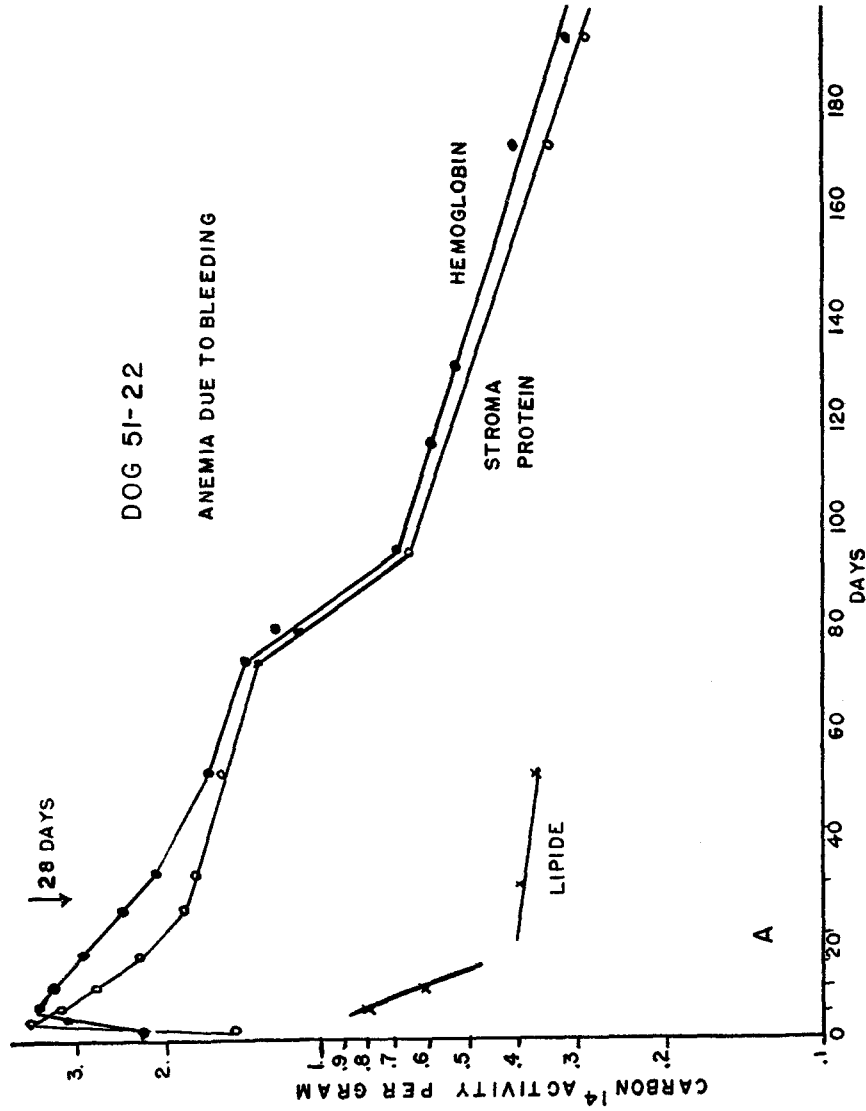


Fig. A. Dog 51-22. Semilogarithmic curves of carbon¹⁴ activity (expressed as arbitrary units each approximating 0.01 microcurie) per gram of hemoglobin, stroma protein, and stroma lipide during and after period of recovery from simple anemia of moderate degree due to repeated bleeding. Return of red cell hematocrit percentage to normal is indicated by the arrow at top. Dog received 64 microcuries of *d,l*-lysine- ϵ -C¹⁴ by mouth.

dog 45-2 with anemia and hypoproteinemia (Fig. C) several days again elapsed between attainment of maximum C^{14} activity per gram of stroma protein and hemoglobin. In these 2 animals, however, the highest hemoglobin activity

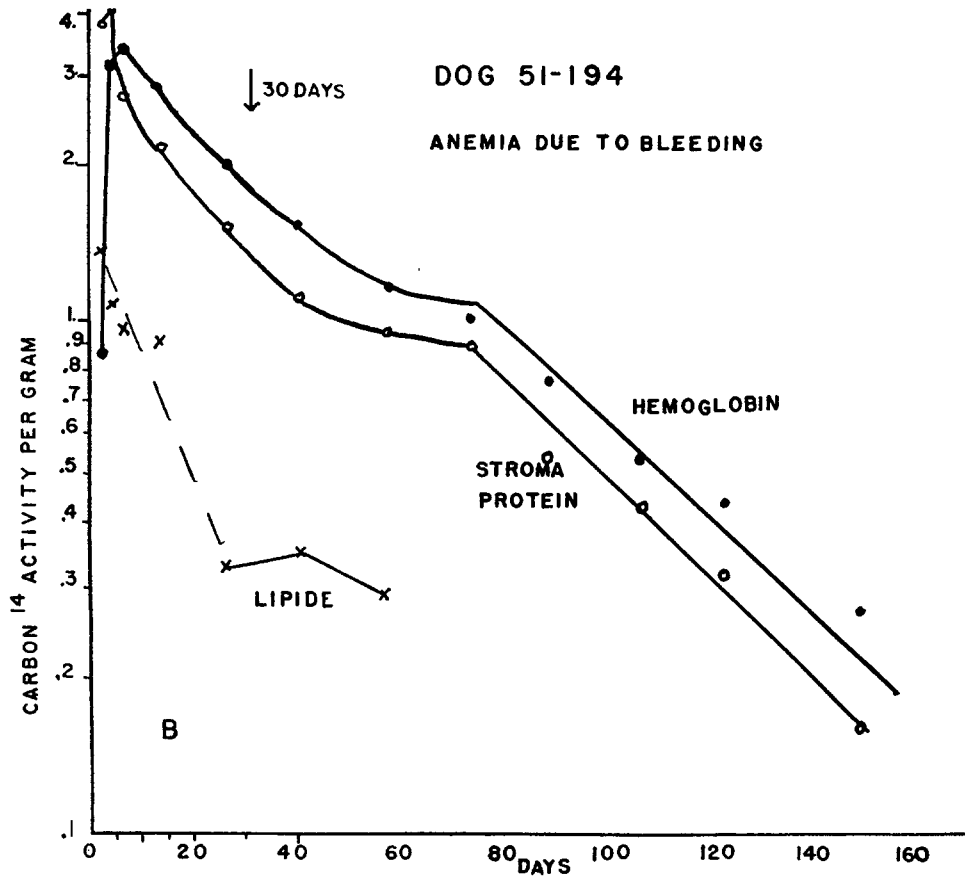


FIG. B. Dog 51-194. Semilogarithmic curves of carbon¹⁴ activity (expressed as arbitrary units each approximating 0.01 microcurie) per gram of hemoglobin, stroma protein, and stroma lipide during and after period of recovery from simple anemia of severe degree due to repeated bleeding. Return of red cell hematocrit percentage to normal is indicated by the arrow at top. Dog. received 87 microcuries of *dl*-lysine- ϵ - C^{14} by mouth.

exceeded the highest value found in stroma protein. This latter finding may reflect an acceleration of hemoglobin production due to the addition of liver extract and lextron plus iron to the regenerating diet fed in these 2 experiments (dogs 47-36 and 45-2) (Table II).

The second phase of Figs. A, B, and C is characterized by a relatively rapid

decline in C¹⁴ specific activity of both red cell protein fractions. During this period of approximately 4 to 6 weeks, corresponding with the time required for the hematocrit percentages to stabilize at normal preanemic levels, the activity of stroma protein decreases at a rate significantly more rapid than does that of hemoglobin.

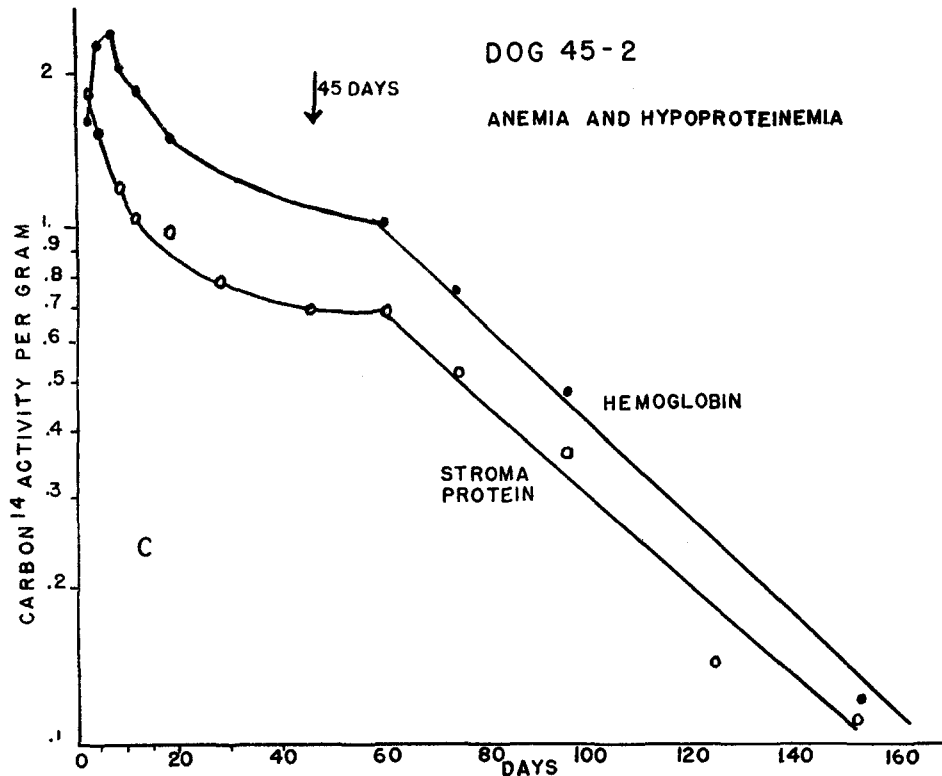


FIG. C. Dog 45-2. Semilogarithmic curves of carbon¹⁴ activity (expressed as arbitrary units each approximating 0.01 microcurie) per gram of hemoglobin, stroma protein, and stroma lipid during and after period of recovery from anemia and hypoproteinemia. Return of red cell hematocrit percentage to normal is indicated by the arrow at top. Dog received 40 microcuries of *dl*-lysine- ϵ -C¹⁴ by mouth.

During phase 3 from about 30 to 80 days in the 3 dogs with simple hemorrhage anemia, including dog 47-36 not charted, and 30 to 50 days in the anemic and hypoproteinemic dog, the rates of decline of C¹⁴ from stroma protein and hemoglobin both become slower. Eventually the disappearance rates become equal as indicated by stabilization of the ratio of hemoglobin to stroma protein activity.

Finally there is an abrupt acceleration of the rates at which C^{14} disappears from both stroma protein and hemoglobin. For the remainder of each experiment, the curves for the 2 red cell fractions are parallel straight lines.

The results shown in Fig. D (dog 46-9) following a period of phenylhydrazine anemia contrast sharply in several respects with those seen after hemorrhage

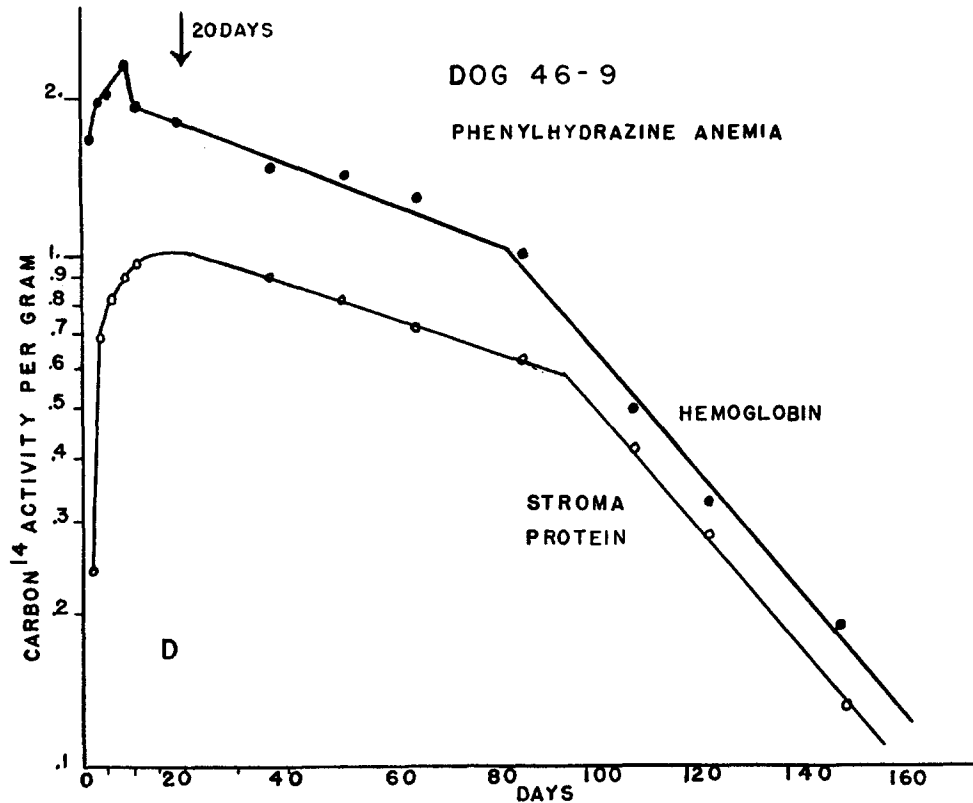


FIG. D. Dog 46-9. Semilogarithmic curves of carbon¹⁴ activity (expressed as arbitrary units each approximating 0.01 microcurie) per gram of hemoglobin, stroma protein, and stroma lipide during and after period of recovery from severe hemolytic anemia produced by multiple injections of phenylhydrazine. Return of red cell hematocrit percentage to normal is indicated by the arrow at top. Dog received 27 microcuries of l-lysine- C^{14} by mouth.

anemia. Hemoglobin C^{14} activity per gram reaches a maximum 9 days after labeled lysine feeding. This maximum is more than double the highest stroma protein value, which is attained at about the 18th day. By this time, some 2 weeks earlier than in all the experiments of other types, the red cell hematocrit percentage had returned to normal. During the next 60 days C^{14} activity dis-

appeared at a similar, relatively slow rate from both stroma protein and hemoglobin. After this period of time the rate of disappearance of C¹⁴ activity hemoglobin was suddenly increased about threefold. Stroma protein activity continued to decline at the slow rate for a further 2 weeks after which the curves for the 2 components again became parallel.

Stroma lipide C¹⁴ activities were of sufficient magnitude to measure accurately only in the first 40 to 50 days of 2 experiments (dogs 51-22 and 51-194). These are illustrated in Figs. A and B. The stroma lipide fraction was labeled

TABLE III
Blood Regeneration after Severe Anemia
Liver Diet Dog 51-194

Days	Red cell hematocrit percentage	Hemoglobin	Total plasma protein	C ¹⁴ activity units per gm.*			
	<i>per cent</i>	<i>gm. per cent</i>	<i>gm. per cent</i>	Stroma lipide	Stroma protein	hemo-globin	Plasma protein
0	17.3						
2	18.5		6.72	1.36	3.77	0.86	7.30
4	19.8	5.26	6.42	1.07	4.38	3.16	5.62
6	21.8	5.75	6.72	0.96	2.72	3.35	4.01
13	38.0	12.1	5.70	0.92	2.18	2.82	2.40
26	47.5	14.5	6.14	0.33	1.51	2.01	1.00
40	50.2	14.4	6.50	0.35	1.11	1.52	0.62
57	48.0		5.85	0.29	0.94	1.17	0.75
73	49.0		6.21		0.88	1.00	0.50
88	52.2		6.43		0.54	0.75	0.47
106	49.5		6.49		0.43	0.54	0.30
122	45.7		6.45		0.32	0.44	0.18
150	49.5		6.10		0.16	0.27	0.14

* 1 unit = approximately 0.01 μ c.

rapidly but to a considerably less extent than either stroma protein or hemoglobin. However, stroma lipide to protein ratios remained relatively constant. The appreciable C¹⁴ activities observed in stroma lipides during the period of regeneration are significant and indicate a conversion of the carbon chain of *L*-lysine into this red cell fraction in the dog.

Plasma protein activities are not recorded in the figures. Disappearance curves were essentially similar to those previously described¹ (1) and were quite independent of those for red cell stroma protein and hemoglobin (Table III). In fact, the plasma protein isotope concentration was falling rapidly as the stroma isotope concentration was rising.

DISCUSSION

The parent red cell in the marrow is a large cell with a relatively large nucleus, clear cell protoplasm, and well defined cell outlines. In its early stages

it contains no evident hemoglobin. Presumably it contains considerable stroma and little or no hemoglobin. The curves in Figs. A and B show that the stroma isotope incorporation reaches its peak in 3 to 4 days, definitely in advance of the peak hemoglobin concentration 2 to 3 days later. The evidence fits in with the assumption that *stroma building precedes hemoglobin construction*.

All 4 dogs with anemia due to blood loss are shown in Table I. The stroma protein C¹⁴ concentration was maximal 2 to 3 days before the peak hemoglobin concentration. In dogs 51-22 and 51-194 the stroma protein C¹⁴ activity exceeded the hemoglobin C¹⁴ activity per gram protein. In dogs 47-36 and 45-2 the maximal C¹⁴ concentration was found in the cell hemoglobin rather than in the cell stroma. This we attribute to the diet which in these 2 dogs contained more liver plus liver extract plus iron in comparison with the first 2 dogs.

The most interesting period in this study fell within the 3 to 5 weeks during which time the regeneration of new red cells brought the red cell hematocrit percentage back to normal. The decreasing concentrations of isotope in the proteins of the red cells varied considerably with the type of anemia.

Phenylhydrazine anemia shows isotope concentration curves (Fig. D) which are quite different from those of the anemia due to blood loss. The hemoglobin isotope concentration curve is not unlike that of the simple anemia experiments, as to the peak levels, but the red cell formation is much more rapid—a return to normal hematocrit percentage in 20 days. The red cell stroma isotope concentration shows a curve which is quite different. Its maximum level is only about one-half that of simple anemia experiments and this maximum is attained only after 10 to 15 days. Meanwhile the stroma protein mass is found to be double that of the simple anemia experiment.

In these phenylhydrazine experiments large amounts of red cells are broken down daily and the pigment radicle is thrown out. Much of the globin, and presumably the stroma protein, and all the iron are saved by the body and stored as reserves in the reticuloendothelial system, organs, and tissues. Under these circumstances with this great mass of unlabeled potential building material, we may assume that much unlabeled stroma protein from body stores (hemolytic products) may dilute the labeled stroma protein formed from the abundant diet intake plus C¹⁴ lysine.

The curves of protein isotope concentration with hypoproteinemia and anemia (Fig. C) are somewhat different from those of the simple hemorrhage anemia. The recovery from anemia and the return to a normal red cell hematocrit percentage require 45 days in sharp contrast with the phenylhydrazine anemia of 20 days. In one experiment we have great surplus stores of protein building materials and in the other the protein reserves have been greatly depleted (hypoproteinemia).

After the peak isotope concentration in the red cell proteins has been reached, the fall is neither as rapid nor as sustained as in Figs. A and B. It can be claimed that this is due to less red cell production and less active production of hemo-

globin and stroma for these cells, consequently less dilution with unlabeled proteins. The delay in recovery of a normal hematocrit percentage and the known tendency of the body to replete its reserve protein stores promptly may explain these curves in Fig. C.

One may note (Fig. C) the sharp break in C¹⁴ activity curves which occurs at 60 days, 2 to 3 weeks earlier than with hemorrhage or phenylhydrazine anemia. This may suggest a shorter life span for the red cell population labeled after a period of anemia and hypoproteinemia.

During the period of active recovery from simple anemia due to blood loss or anemia plus hypoproteinemia both stroma protein and hemoglobin C¹⁴ activity decline rapidly. This is presumably due to continuing regeneration with influx of relatively unlabeled cells into the circulation. It is of interest, however, that in all these dogs hemoglobin activity declined more slowly than did stroma protein activity until the hematocrit percentage stabilized. This finding is probably related to continuing synthesis of hemoglobin, from partially labeled sources, by reticulocytes in the peripheral blood (3).

It is apparent from the foregoing discussion that under different experimental conditions, various factors modify the C¹⁴ activities of red cell hemoglobin and stroma protein, with regard to uptake and disappearance, prior to complete recovery from anemia. Thereafter during the remainder of the life span of the red cell, the isotope concentrations of the 2 erythrocyte proteins decline at identical rates as shown by the parallel portions of the curves in Figs. A, B, C, and D. This indicates that between complete maturation and final breakdown, the red cell acts as an intact stable unit in so far as hemoglobin and stroma are concerned.

SUMMARY

Red cell stroma protein and hemoglobin can be labeled by feeding C¹⁴ lysine during periods of active blood regeneration following anemia.

Stroma proteins are produced and a maximum concentration of the C¹⁴ label appears 2 to 3 days earlier than with hemoglobin,—which is to say that stroma building precedes hemoglobin construction.

The concentration of isotope in stroma protein may exceed its concentration in hemoglobin during regeneration following anemia due to blood loss. Diets favorable for hemoglobin regeneration may force the hemoglobin isotope concentration above that of the stroma protein.

In hemolytic anemias great reserves of red cell building material are stored in the body. These stores may modify the curves of isotope concentration in red cells during the recovery periods.

When finally formed, the mature red cells show little or no evidence of participation in general body protein metabolism during their life in the circulation.

BIBLIOGRAPHY

1. Van Slyke, D. D., Steele, R., and Plazin, J., *J. Biol. Chem.*, 1951, **192**, 769.
2. Bale, W. F., Yuile, C. L., DeLaVergne, L., Miller, L. L., and Whipple, G. H., *J. Exp. Med.*, 1949, **90**, 315.
3. Nizet, A., and Robschuit-Robbins, F. S., *Blood*, 1950, **7**, 648.
4. Pappenheimer, A. M., and Hendee, E. D., *J. Biol. Chem.*, 1949, **180**, 597.
5. Sumner, J. B., and Summers, G. F., *Laboratory Experiments in Biological Chemistry*, New York, Academic Press, Inc., 1944.
6. Terry, R., Sandrock, W. E., Nye, R. E., Jr., and Whipple, G. H., *J. Exp. Med.*, 1948, **87**, 547.
7. Tishkoff, G. H., Robschuit-Robbins, F. S., and Whipple, G. H., *Blood*, 1953, **8**, 459.
8. Robschuit-Robbins, F. S., Miller, L. L., and Whipple, G. H., *J. Exp. Med.*, 1943, **77**, 375.