Relation of Porphyrin Content to Red Cell Age: Analysis by Fractional Hemolysis[1] (36107)

LaRue W. Johnson and Samuel Schwartz[2]

Department of Veterinary Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota 55101, and Department of Medicine, College of Medical Sciences, University of Minnesota, Minneapolis, Minnesota, 55455

The present studies were undertaken to determine how long uroporphyrin (uro), coproporphyrin (copro), and protoporphyrin (proto) persist in circulating erythrocytes. Synthesis of these porphyrins is limited essentially to the mitochondria-containing erythroblasts and reticulocytes. While indirect evidence has indicated that uro and copro probably persist for up to one week, or slightly more, the persistence time of protoporphyrin is quite uncertain; it has been estimated to range under various conditions from that of the reticulocyte to that of the mature red cell (1, 2).

This problem might, theoretically, be resolved by isotopic analysis of erythrocyte porphyrins isolated at intervals following the injection of a precursor such as glycine-^{14}C. However, even subjects with erythropoietic ("congenital") porphyria generally have concentrations of erythrocyte uro and coproporphyrin of less than 20 μg/100 ml of cells. The volumes of blood required would therefore be prohibitive, even for cattle with this condition. Alternative procedures were therefore explored (3).

An earlier report (4) has described the use of fractional lysis in hypotonic saline to separate bovine erythrocytes of different ages. Marked differences in specific activities of hemoglobin-protoporphyrin (Hb-proto)-^{14}C were found in the successive lysates obtained from bloods tested at intervals after injection of glycine-2-^{14}C. This suggested the possibility that approximate "half-times" could be calculated for each porphyrin (or for any unlabelled compound) found in the same lysate fractions by comparing its relative distribution with that of Hb-proto-^{14}C as considered below.

Materials and Methods. Protocol of study. Porphyric heifer No. 2966 had been protected from direct sunlight since birth and throughout this study, begun at 6 months of age. She was bled repeatedly to increase erythrocyte porphyrin concentration. Details of the method employed for fractional hemolysis of her erythrocytes are described in the earlier report (4) along with isotopic data obtained for her Hb-proto. Additional information is given in the section on results, below.

Porphyrin analyses. Ten ml (or more) of each hemolysate fraction were removed for isolation of Hb-proto (5). The remaining hemolysate was added to 12 volumes of a 4:1 mixture of ethyl acetate:glacial acetic acid, and the uro, copro, and proto separated for quantitative fluorimetric analysis (6).

Results. 1. *Hematology and erythrocytic porphyrin data.* With repeated phlebotomy and a reticulocytosis of 4–6%, the concentrations of uro and copro rose from levels of less than 10 μg/100 ml to an average of 114 and 18 μg/100 ml respectively during the first 2 weeks following injection of the glycine-^{14}C (Table I). From days 19 to 120, with diminished blood loss and decreased erythropoietic stimulation, these average values fell to 27 and 8.5 μg/100 ml respectively. Red cell proto values remained elevated above 600

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TABLE I. Calf 2966: Hematology and Porphyrin Data.

Days[a]	Retics (%)	Hb (Gm %)	RBC Porphyrins, μg/100 ml		
			Uro	Copro	Proto
(—) 21	0.3	6.9	9	9	661
(—) 14[b]	5.1	6.9	26	12	786
(—) 9	—	6.6	82	24	615
(—) 1	5.9	6.2	102	14	638
2	6.0	7.3	96	26	475
4	4.6	7.4	161	19	408
8	5.5	6.9	107	17	313
19	2.7	7.3	43	20	494
40	3.6	8.5	40	6	270
83	1.4	8.5	26	11	355
120	0.6	8.4	10	8	500

[a] Seven phlebotomies, 500 ml each, begun Oct. 21, 1963, 23 days before injection of glycine-2-^{14}C. (Injection on day 0).

[b] One gram of iron dextran injected intramuscularly; 5th phlebotomy. To conserve space, data are included from only 4 of 7 pre-injection and 7 of 12 post-injection blood studies.

μg/100 ml during the period of phlebotomy. They fell sharply following isotope injection, possibly in association with the iron dextran given im on day (—) 14.

Mean corpuscular hemoglobin concentration (not shown) remained at about 33–34%, while control mean corpuscular hemoglobin values of 10.2 and 10.7 μμg rose progressively to about 15 by day 120 (3). Average red cell survival was approximately 50 days (7). The moderately severe anemia found even prior to phlebotomy (and despite the absence of significant light exposure) is also to be noted.

2. *Absolute and relative distribution of Hb and other porphyrins in the 4 successive hemolysate fractions.* The average distribution (% of total) of Hb, and of uro, copro, and proto in each of the 4 hemolysate fractions is given in Table II, part A. The rela-

TABLE II. Distribution of Hb and Free Porphyrins in 4 Successive Lysate Fractions.

	Fraction I		Fraction II		Fraction III		Fraction IV	
	Av	SD	Av	SD	Av	SD	Av	SD
A.	Percent of total							
Analysis								
Hb	9.1	(1.8)	23.9	(4.2)	43.9	(1.7)	22.0	(4.5)
Uro	37.5	(4.2)	29.7	(8.1)	24.6	(4.3)	8.9	(4.3)
Copro	16.5	(4.4)	27.0	(8.6)	37.9	(6.6)	18.5	(6.6)
Proto	8.3	(2.4)	24.1	(4.9)	48.7	(4.5)	18.8	(5.1)
B.	Relative Amounts; % of Total, Porphyrin/Hb							
Ratios								
Uro/Hb	4.13		1.24		0.56		0.40	
Copro/Hb	1.81		1.13		0.86		0.84	
Proto/Hb	0.91		1.01		1.11		0.85	

Average values were calculated from mg quantities of each compound found in the 4 hemolysates of 12 blood samples drawn 2–120 days after injection of glycine-2-^{14}C. Cell fragility is greatest in fraction I, least in fraction IV (4).

tive distribution of each porphyrin is compared to that of Hb in the same fractions in part B of this table.

Gross inspection of the data in Table II shows that the "free" porphyrins differed markedly from one another in their distribution among the 4 hemolysate fractions. Uro, and to a lesser extent copro, was concentrated in the hemolysate of the most fragile cells, fraction I. Since similar concentration of Hb-proto-^{14}C was found (4) only for cells which were less than 1 week of age, these 2 porphyrins must likewise have been present mainly in cells which were less than 1 week of age. A more prolonged persistence of proto, however, is indicated by the similarity of its distribution to that of Hb in each of the fractions.

3. *Factors considered for calculation of "half-times" of chemical constituents of circulating erythrocytes.* Two sets of data must be correlated—first, the distribution found for any compound in the successive hemolysate fractions (*i.e.*, porphyrins as in Table II), and secondly, the known distribution of cells of different ages determined simultaneously in the same fractions. The latter information was illustrated in Fig. 1 of the earlier report (4) and is reproduced here in part, as Fig. 1-A. Since the large majority of initial labelling is complete within one day after injection of the glycine-2-^{14}C and since the label tested (^{14}C in Hb-proto) persists essentially for the life time of the cell, we may consider the isotope label found in any fraction to represent only cells whose age is equal to the interval in days since the isotope injection. The distribution of total (unlabelled) uro, copro, and proto in circulating erythrocytes, however, is quite unlike that of Hb-proto-^{14}C since concentrations of porphyrin reflect two phenomena; first, a *cumulative* effect due to constant liberation into the circulation of new porphyrin-containing cells, and secondly, a *diminution* effect due to loss of porphyrin from intact cells during cell aging. These two phenomena in calf 2966 can be analyzed separately.

a) *Cumulative values* for each fraction were determined from the Hb-proto-^{14}C data (Fig. 1-A), by calculating values which would have been obtained under similar conditions had daily injections of glycine-2-^{14}C been made in this animal. Thus for any given day (after day 2) the % of Hb-proto-^{14}C in any fraction should be equal to the sum of the daily percentage values found (or extrapolated from Fig. 1-A) for that fraction, divided by the number of additional injection days. Results of such calculations are shown in Fig. 1-B. The increasing differences between the calculated cumulative values in this figure (1-B) and the experimental values found for the "single population" of labelled cells (Fig. 1-A) are apparent. The general validity of these calculations is confirmed by the fact that the average (mg) amounts of hemoglobin actually found in each of the 4 lysate fractions (right side, Fig. 1-B) agreed well with those predicted in Fig. 1-B. Since these calculated values remain fairly constant after the first month, the values found would be consistent with a red cell survival of about 30–120 days. The average survival time found was 50 days (7).

b) The modifying effect of *porphyrin loss* from intact cells may be tested for two major possibilities, *i.e.*, that the porphyrin disappears rapidly after some definable period, or that it disappears at essentially exponential rates. If the first possibility were true, the percentage of "free" porphyrin found in each fraction would correspond to the cumulative values calculated for Hb-proto-^{14}C (Fig. 1-B) for some particular day. The poor fit of observed experimental values, (especially those of protoporphyrin) and of those calculated for any day suggest that this is quite unlikely.

4. *Calculations based on exponential loss of free porphyrins from circulating erythrocytes.* These calculations were made as for Fig. 1-B, except that each experimental or extrapolated value from Fig. 1-A was corrected for daily losses predicted for any given half-time. Calculated theoretical values for compounds with half-times of 1.5–20 days are shown in Fig. 2-A. The 4 average lysate values found experimentally for uro were almost identical to those predicted for compounds with a half-time of 1.9 days (Fig. 2-B). The similar agreement of values pre-

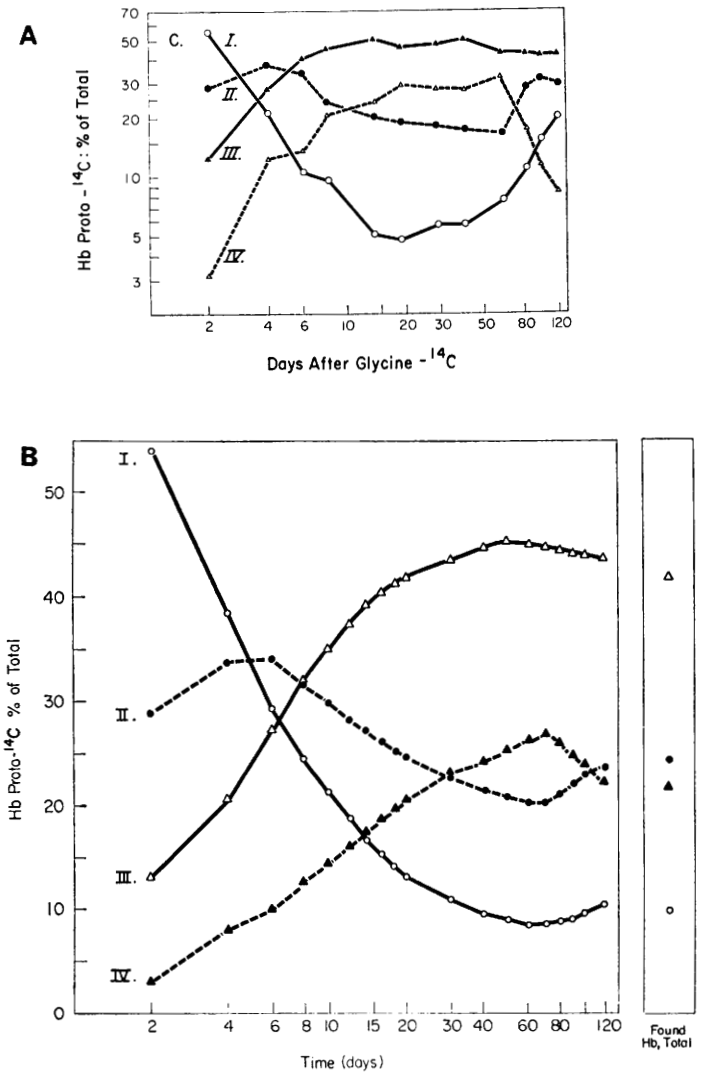

FIG. 1. Hb-proto-^{14}C, % of total in each of 4 successive hemolysate fractions: A. Experimental values found, calf 2966. Blood samples drawn 2–120 days after a single dose of glycine-2-^{14}C. (Ref. 4). B. Calculated cumulative values predicted for similar daily isotope injections. Experimental average values of Hb found in 12 samples shown for comparison.

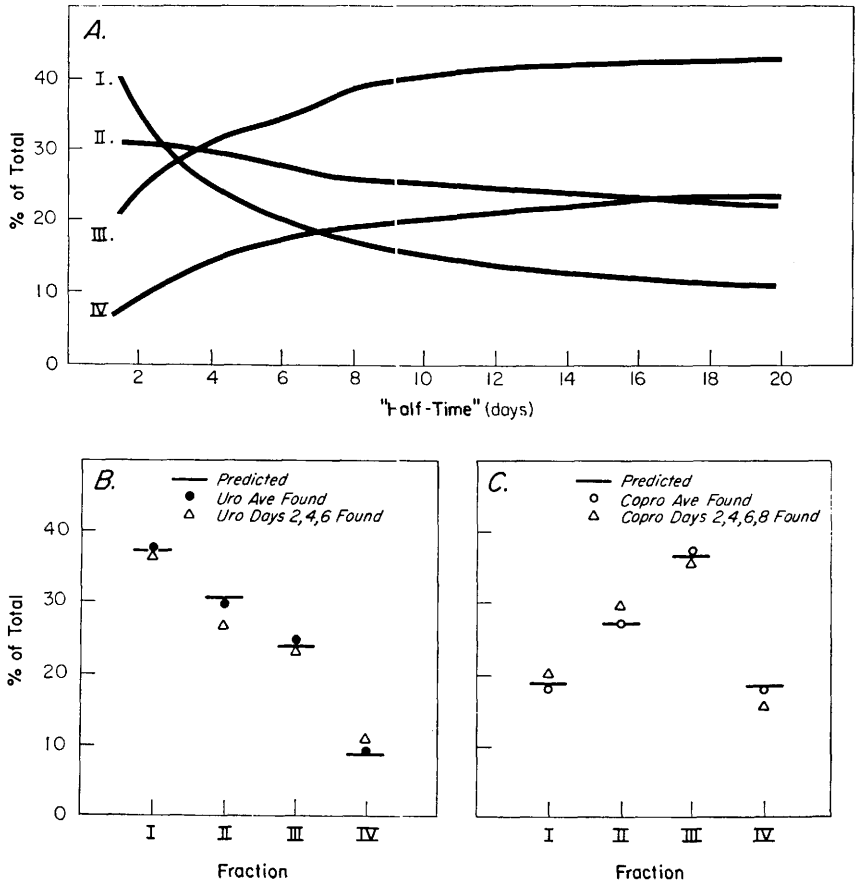

FIG. 2. Determination of half-times of porphyrins in circulating erythrocytes. A. Calculated cumulative distribution of any compound in 4 successive hemolysate fractions, where these compounds are lost exponentially with half-times of 1–20 days. B. Distribution in 4 hemolysate fractions of a compound with a half-time of 1.9 days. Predicted values are compared with averages of all the values found for uro as well as with those found only during the more critical 2–6 day period. C. Same as B, values for any compound with a half-time of 7 days. Predicted values compared with those found for copro in the 4 hemolysate fractions.

dicted for a 7 day half-time and those found for copro is also seen (Fig. 2-C). The average values found for protoporphyrin in the 4 hemolysate fractions do not fit any projected time shown in either Fig. 1 or 2. Good fits, however, were obtained by assuming that the proto content of young erythrocytes first increased for several days after their release from the bone marrow and then underwent exponential loss. Though such calculations cannot be decisive enough to warrant detailed consideration here, they are consistent with several findings to be considered below.

Discussion. In animals or humans with erythropoietic porphyria (8) or in experimental animals treated with lead, plenylhydrazine, or low oxygen tension (9, 10), the concentrations of uro and of copro are much higher in marrow than in circulating blood. Similarly, in circulating erythrocytes, the concentration of copro correlates well with the reticulocyte percentage (11–14). Neither of the above findings are true of proto. In addition, uro is found in circulating cells mainly in association with increased numbers of normoblasts or other evidence of rapid

cellular release from marrow. These observations are consistent with a very short half-time for uro (1.9 days calculated here) and of a somewhat longer half-time for copro (7 days calculated here). They also suggest that a major portion of the erythrocyte (free) protoporphyrin is synthesized in more mature cells than is true of the uro and copro, *i. e.*, in reticulocytes. Under these conditions one would not expect the proto distribution in the 4 hemolysate fractions to fit predictions for any half-time, as calculated earlier.

The above conclusion for proto is strengthened by recent *in vivo* and *in vitro* studies of a patient with (erythropoietic) protoporphyria (15) which found that the accumulation of erythrocyte protoporphyrin in this condition is most likely due to a selective defect in reticulocytes (and presumably in late-stage normoblasts) which show a relative increase in mitochondrial ALA-synthetase and decrease in heme synthetase activities. In the same study, isotopic data indicated the presence of several distinct pools of protoporphyrin, with half-times ranging from an hour or less to 12 days. The large majority of circulating proto was in the "12 day" pool.

Evidence for half-times of erythrocyte proto may be obtained where the underlying disease is treated effectively. Thus, in patients with iron deficiency anemia, values fall within 2–3 weeks (or less) to about ½ the pretreatment values, generally after a lag of about 3–7 days (2, 14, 16). This lag phase and the persistence for 2–4 weeks of a relatively constant rate of decline in porphyrin concentration with half-times of about 10–15 days both suggest that the administered iron does not act by complexing already existing erythrocyte proto, but rather prevents accumulation of newly formed proto by permitting complete heme synthesis in immature cells. Related data was reviewed in 1940 by Seggel (17), who summarized earlier studies of the relationship of fluorocytes (red blood cells which fluoresce red on exposure to near-ultraviolet light), reticulocytes, and proto content in a variety of conditions. While fluorocyte counts were not strictly quantitative, their numbers generally correlated well with total proto content. Where both fluorocytes and reticulocytes were increased, reticulocytes tended to be fluorocytes, but most fluorocytes were not reticulocytes. With a single erythropoietic stimulus or with therapy as discussed above, changes in fluorocyte counts were most consistent with half-times of approximately 10–20 days.

Finally, it should be emphasized that half-times of erythrocyte porphyrins are undoubtedly not uniform under all conditions, and that a single half-time does not describe adequately the behavior of what may be separate pools with different half-times (15). It would seem most likely that each porphyrin (like reticulocytes themselves) has a range of half-times which reflect variations in normal and abnormal metabolic states.

Summary. Procedures are described for the calculation of approximate half-times of unlabelled porphyrins (or of any compound) in circulating erythrocytes. These are based on a comparison of their distribution in fractionally lysed erythrocytes and the distribution within the same hemolysate fractions of cells of different ages. Cell age was determined by cohort labelling of new-formed hemoglobin following injection of glycine ^{14}C or ^{59}Fe. Analysis of the distribution of uro, copro, and protoporphyrin in such hemolysates from a porphyric cow indicated that half-times of her erythrocyte uro and coproporphyrin were about 2 and 7 days, respectively, while erythrocyte protoporphyrin accumulated for several days in circulating reticulocytes before decreasing in concentration. Data favoring a half-time of 2–3 weeks for the major portion of erythrocyte protoporphyrin are cited.

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