Differential Lysis of Bovine and Canine Erythrocytes in Saline Versus Saponin: Relation to Cell Age[1] (36009)

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Marked differences in osmotic fragility have been reported for erythrocytes of different species (1). In humans (2–4), rats (5), and rabbits (6, 7) most young erythrocytes are more resistant to lysis in hypotonic saline than are older cells, though varying proportions of these young cells may show increased osmotic fragility. Studies of porphyrin content of lysates of red cells from two porphyric cows (8), however, were believed to indicate a uniquely increased osmotic fragility of most young erythrocytes in these animals. Following injection of ^{59}Fe citrate in 4 acutely bled dogs (9, 10), young erythrocytes were found to be relatively more fragile than average. Such increased fragility was less certain in the single normal dog studied.

The present studies (11) in cattle and dogs had 3 goals: (i) to investigate directly and in greater detail the relationship of cell age to osmotic lysis in these 2 species; (ii) to do comparable studies with potentially more useful chemicals, such as saponin; and (iii) to relate the concentration of various constituents of successive hemolysate fractions to the half-times of these constituents in the circulating cells. Analyses dealing with the latter application are considered elsewhere (12).

Materials and Methods. Protocols of animal studies. Studies have been made of erythrocytes from 1 porphyric and 3 normal Holstein-Friesian cattle, all 3–8 months of age, and from 2 normal adult dogs. Single intravenous injections of glycine-2-^{14}C (9–17 μCi/kg) and/or of ^{59}Fe-citrate (0.24–2.1 μCi/kg) were given to each. The most detailed studies were made of porphyric heifer No. 2966 in which seven phlebotomies of 500 ml each were performed over a 20 day period to stimulate erythropoiesis prior to injection of the glycine-2-^{14}C. Twelve blood samples of 140 ml each (heparinized) were removed subsequently, from days 2 to 120. Such phlebotomies were not performed in any of the other animals.

Studies of *in vivo* red cell survival and other hematology data in the cattle are described elsewhere (13).

Preparation of hemolysates. Fractionation of hemolysates employed a serial osmotic fragility technique similar to that described by Simon and Topper (2). Four or 5 successive hemolysate fractions were prepared in each study. The concentrations of NaCl (or saponin) employed were determined in preliminary tests to define conditions which would yield approximately 10% of the total hemoglobin (Hb) in the first and last lysate fractions. These conditions were found to vary among different individuals, but were essentially as follows in the cattle: The heparinized blood was cooled immediately in an ice bath, and kept at about 4° throughout the preparative procedure. The centrifuged cells were washed 3 times with isotonic saline, suspended in 5 vol of 0.51 to 0.60% NaCl, and kept at 4° for 10 min. Following centrifugation, the supernatant solution (fraction I) was removed by aspiration. The remaining cells were then hemolyzed by suc-

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cessive similar treatment with the same volumes of progressively lower concentrations (0.52 to 0.44%) of NaCl, and finally with distilled water (fractions II–V). Because of the greater resistance of dog erythrocytes to saline lysis (1), initial concentrations of NaCl for their cells ranged from 0.38 to 0.45% for fraction I, and from 0.34 to 0.40% for fraction IV. Again final lysis (fraction V) was with H_2O.

Saponin, dissolved in isotonic saline, ranged from 0.1 to 0.25% concentrations in fractions I–IV in cattle, and from 0.01 to 0.02% in the same fractions in dogs. Final hemolysis was again with H_2O. In each case, 5 vol were added per initial volume of packed cells.

No attempt was made to maintain a constant pH in the lysed samples. The pH in each of the bovine samples tested was 6.0 or less in the first hemolysate, while that of the dog samples generally began at close to pH 6.7. In all cases the pH tended to fall in successive lysates, reaching about 5.6 and 6.2 in the bovine and dog bloods, respectively.

Chemical and isotopic analyses. The Hb concentration and content were determined in each fraction, and the Hb-protoporphyrin (Hb-proto) isolated by Grinstein's method (14) for ^{14}C analysis in a gas flow proportional counter. The ^{59}Fe activity was determined from 4 ml aliquots of each lysate fraction, counted directly for a total of at least 10,000 counts in a well scintillation counter.

In the tests we considered isotope labeling to be essentially complete within 24 hr. Under these conditions, the isotope content of any fraction would, essentially, represent Hb from lysed cells whose age was equal to the number of days elapsed from the injection date.

Results. 1. Studies of porphyric heifer No. 2966: Anemia and stimulated erythropoiesis on the day of isotope injection were reflected in the Hb concentration of 6.6 g/100 ml, and in the 6 reticulocytes found/100 red cells. Results for the 4 successive hemolysate fractions from each of the 12 bloods sampled are summarized in Fig. 1 in terms of the percentage of the total Hb which was found in each fraction (part A), the relative specific activities of Hb-proto in each fraction (part B), and the percentage of total Hb-proto-^{14}C activity found in each fraction (part C). Time is plotted on a log scale to show more clearly the rapid changes which occurred during the first 2 weeks.

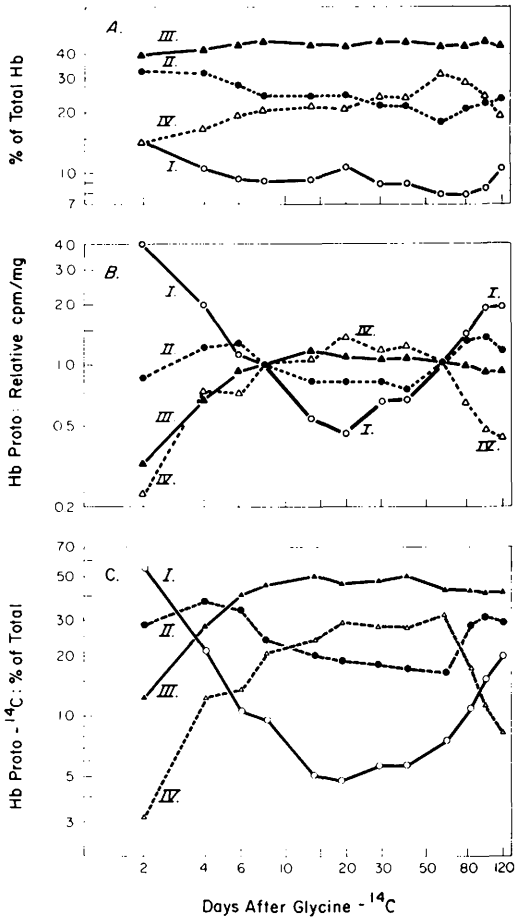

FIG. 1. Distribution of total Hemoglobin and of ^{14}C in Hb-proto in 4 successive hemolysate fractions, days 2–120 after injection of glycine-2-^{14}C. The relative activity (cpm/mg of Hb-proto) is defined here as being equal to the amount (cpm/mg) in any fraction divided by the average (cpm/mg) of all the fractions.

Of major significance is the finding (part B) that very young and, to a lesser extent, very old cells were both most susceptible to lysis in hypotonic saline. Thus, on day 2, the specific activity of the most fragile fraction (I) was 4 times that of the average, or 17 times that of the most resistant fraction

TABLE I. Changes in Isotope Labeling in Hemolysate Fractions Following Injection of ^{59}Fe-Citrate and ^{14}C-Glycine; Normal Calf 3301.[a]

Day	Label	Relative activity fractions I–V		
		I	III	V
3	^{59}Fe	5.0	0.4	0.6
	^{14}C	8.3	0.3	0.6
10	^{59}Fe	2.2	0.5	0.7
	^{14}C	2.2	0.5	0.7
14	^{59}Fe	1.4	0.8	0.7
	^{14}C	1.3	0.8	1.0
20	^{59}Fe	1.0	0.8	0.8
	^{14}C	1.0	0.8	1.0
42	^{59}Fe	0.8	1.0	1.1
	^{14}C	0.7	1.1	1.0
65	^{59}Fe	0.8	0.9	1.5
	^{14}C	1.0	0.5	1.0
84	^{59}Fe	1.3	0.8	1.2
	^{14}C	0.9	0.9	1.0
105	^{59}Fe	1.8	0.8	1.1
	^{14}C	1.9	1.0	0.6

[a] To simplify this table (and Table II), values for fractions II and IV are omitted. They were, in almost every instance, intermediate between those of adjacent fractions. Relative activity is defined as for Fig. 1B.

(IV). With increasing cell age, the relative specific activities in all 4 fractions rapidly approached one another and were all approximately equal on day 8. Thereafter values diverged temporarily; 2 to 4-week-old cells became more resistant to hemolysis than the average, as shown by the maximum specific activity in fraction IV and minimum activity in fraction I. Finally, hemolysis was again reversed, with Hb-proto-^{14}C from older cells (70 days or more) becoming most concentrated again in the first most fragile fraction.

2. Studies in normal calf 3301 injected simultaneously with ^{14}C and ^{59}Fe labels. The saline fragility patterns found with each isotope in this normal calf (Hb, 12.3 g/100 ml) were generally similar to those of the anemic porphyric calf for several weeks (Table I). The consistently greater relative activity of ^{59}Fe in fraction V from days 65–105 reflects reutilization of ^{59}Fe, but not of ^{14}C (13). Comparable results, not included here, were obtained for Hb-proto-^{14}C in hemolysates of normal calf 3078, studied only on days 6, 16, 28, and 104 (11). Additional data are given in Table II.

3. Comparison of saponin and saline as lytic agents in cattle and dogs. Comparative studies of these 2 agents are summarized here only for the critical first week (Table II). Young cells were most fragile with both agents in the cattle, minor differences being due to differences in Hb content (not shown) in the corresponding fractions. In the dogs, on the other hand, striking differences were found; the young cells were most resistant in hypotonic saline, but most fragile in saponin. Older cells in the dog (days 57–115) showed increased osmotic fragility.

As noted earlier, bovine erythroctes were much more fragile in hypotonic saline than

TABLE II. Similarity of Saline and Saponin Lysis of Normal Bovine Erythrocytes Contrasted with Their Differing Behavior Toward Canine Erythrocytes.[a]

Lytic agent	Day	Relative activity (^{59}Fe) fraction		
		I	III	V
		Bovine study		
Saponin	2	13.7	0.8	0.3
Saline		12.0	0.5	0.8
Saponin	3	4.5	0.8	4.0
Saline		5.0	0.4	0.6
Saponin	6	5.0	0.7	1.2
Saline		3.3	0.8	0.8
		Canine study		
Saponin	2	2.1	0.4	0.2
Saline		1.4	0.4	3.5
Saponin	3	1.3	0.9	0.4
Saline		0.7	0.3	4.2
Saponin	6	0.9	1.5	0.4
Saline	7	0.4	1.2	1.4
Saline	8	0.2	0.9	1.3
Saline	57	1.5	0.9	0.6
Saline	91	1.9	1.0	0.8
Saline	115	2.6	1.0	1.3

[a] Bovine studies: Normal cow, 3256, except for day 3 saline data from cow 3301. Dog studies: Saponin data (and day 3 saline data) from dog number 3; remaining saline data from dog number 2.

were those of dogs. The reverse was true with saponin, where only about 1/10 as much saponin was required for hemolysis of canine cells as compared to those of cattle.

Discussion. The techniques employed here (from 1962–1965) for fractional red cell hemolysis were rather crude compared to some very elegant methods described in recent years. These latter methods have been reviewed by Van Gastel (15). Some have employed osmotic lysis (2–5, 16–18) others immune hemolysins (19, 20) or chemicals such as primaquine (21). The role of modifying factors such as pH (18) or addition of materials such as nucleosides (20) have also been described. With nearly all these methods, the older cells have been reported to show the greatest susceptibility to lysis, with variable lysis of young cells. Though Borek *et al.* (19) found young red blood cells of the rat to be most susceptible to lysis by rabbit hemolytic factor, old cells were found most fragile in similarly treated blood from humans and dogs (22) and from sheep (20).

Frei *et al.* (23) have prepared osmotic fragility curves (fragiligraphs) of cattle. Until the eighth or ninth week after birth, bimodal curves were found, with fetal hemoglobin present almost entirely in the osmotically more resistant fraction. The first, more fragile, fraction was characteristic of mature cattle, such as those included in our study. Bimodal curves were also reported in newborn pigs, rabbits, mice, hamsters, and guinea pigs (1). In neither report, however, was there any suggestion that differential lysis related directly to red cell age.

Since primary interest usually relates to separation of young cells or their contents, and since it is easier to isolate the first (*i.e.*, 10%) portion of hemolysate than the intermediate or final portions, such studies are technically more feasible in the bovine than in other species reported, using either osmotic or saponin lysis. The studies reported here also suggest that use of saponin, rather than of saline, offers the advantage of most rapid lysis of young cells in dog blood. The possibility that this might be true also of blood from humans and other species has not been tested. The potential application of data from successive lysates for direct determination of half-times of red cell constituents is considered elsewhere (12).

Summary. Very young erythrocytes in cattle (normal or porphyric) appear to be relatively unique in showing markedly increased fragility in hypotonic saline. Most young erythrocytes in a normal dog, in contrast to earlier reports of acutely bled animals, were the most resistant to osmotic lysis. Saponin preferentially lysed young erythrocytes in a normal dog, as well as in a normal calf, and may thus have special value for preparation of hemolysates enriched in constituents of young red cells. Cell age in all studies was established by a single intravenous injection of glycine-2-^{14}C or of ^{59}Fe-citrate; and age-dependent fragility determined by suitable isotope analyses of successive hemolysate fractions was carried out on bloods sampled periodically for up to 120 days.

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