AGE AS AFFECTING THE OSMOTIC AND MECHANICAL FRAGILITY OF DOG ERYTHROCYTES TAGGED WITH RADIOACTIVE IRON*

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Radioactive iron administered intravenously rapidly becomes a part of new hemoglobin and appears in the circulating red cells in a few hours, thus effectively tagging the newly formed cells. When utilization of the administered iron has been maximal and presumably no more is being incorporated in hemoglobin, there exists in the circulation a group of tagged red cells of approximately known age. As the tagged cells mature and become older their fragility can be compared with that of the total cell population by making appropriate measurements,—a test first used by Cruz, Hahn, Bale, and Balfour (6) who showed that young cells are less resistant to hemolysis in hypotonic solutions of sodium chloride than are cells of average age.

The experiments described in the present paper and in an earlier report (24) confirm the findings of Cruz *et al.*, and show in addition that the mechanical fragility of the newly formed dog cells containing radioactive iron is less than that of the general cell population whereas toward the end of their life span it is greater. The increasing susceptibility of older cells to lysis by trauma will be discussed in relation to the events which may determine the life span of erythrocytes.

A decrease in circulating radioactive iron was observed in each of these experiments at a time when the rate of elimination of tagged cells from the circulation was presumably maximal. This unexpected finding has made possible estimates of the life span of dog red cells which agree with those given by other methods.

Methods

The dogs used were all normal mongrels. All had been vaccinated against distemper, and all were fed a diet of hospital table scraps.

Blood samples were withdrawn in clean syringes lubricated with mineral oil and the usual precautions were taken to avoid artificial hemolysis. Heparin was used as an anticoagulant.

The procedures for determination of radioactivity were essentially those described by

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Hahn (9) with modifications as reported by Yuile *et al.* (28). A mixture of Fe^{55} and Fe^{59} obtained from the Atomic Energy Commission was administered intravenously in gelatin (10).

Determinations of Osmotic Fragility of Dog Erythrocytes.—The resistance of the cells was determined by mixing 0.5 ml. of heparinized blood with 5.0 ml. of unbuffered solutions of sodium chloride. The heparin used was liquaemin (Roche-Organon) to the amount of 0.1 ml. (1.0 mg.) per 10 ml. of blood. The final pH of the blood-saline mixtures was approximately 7.4. After an interval of 15 minutes, the tubes were centrifuged for 10 minutes at 1500 R.P.M. The supernatant fluid in each tube was removed and its hemoglobin content measured with the aid of a photoelectric colorimeter. Per cent hemolysis at each tonicity was calculated in relation to the hemoglobin liberated in the tube containing distilled water.

Determinations of Mechanical Fragility of Dog Erythrocytes.—The method of Shen, Castle, and Fleming (22), which subjects red cells to trauma by rolling glass beads, was modified to meet the requirements of Experiments 1 and 2. The rotator employed for the mechanical fragility test has been described elsewhere (25). A 1.0 ml. sample of whole heparinized blood was placed in an Erlenmeyer flask containing 10 glass beads, each of which was 4.0 mm. in diameter. Duplicate flasks were rotated at a rate of 28 R. P.M. for 90 minutes. A similar sample of blood was allowed to stand at room temperature for the same length of time in order to provide an estimate of autohemolysis occurring without trauma. A 0.5 ml. sample of each of the rotated aliquots was then pipetted into 5.0 ml. of a 1.25 per cent solution of sodium chloride to determine the amount of hemolysis induced by trauma. Centrifugation and hemoglobin determination were carried out in the same fashion as for the osmotic fragility test. The per cent hemolysis was calculted as follows:—

$$\frac{B - A}{C - A} \times 100 = \text{per cent hemolysis}$$

where B = free hemoglobin present in rotated sample (averge of the 2 aliquots),
A = free hemoglobin present in standing sample,
C = total hemoglobin liberated by distilled water.

The washed cells employed in the mechanical fragility tests of Experiment 3 were prepared by a modification of Hamilton's technique for handling dog blood (13). The heparinized blood was chilled immediately after being drawn and was centrifuged for 2 minutes at 2500 R.P.M. The plasma was removed and the cells were washed twice, as rapidly as possible, with ice cold isotonic saline, with centrifugation for 2 minutes at 2500 R.P.M. A 1 ml. aliquot of the packed cells (hematocrit approximately 70 per cent) was placed in each of two Erlenmeyer flasks containing 20 beads of 4.0 mm. diameter and spun for 90 minutes at 60 R.P.M. The liberated hemoglobin was measured as previously described.

The unlysed red cells from each tube used in measurements of both osmotic and mechanical fragility were washed twice with a 1.25 per cent solution of sodium chlorice. These cells, as well as aliquots of the supernatant solutions, were ashed, plated, and measured for radioactivity. Each determination of radioactivity was thus made in duplicate—one sample representing released hemogobin and the other the hemoglobin retained in unlysed cells. As additional checks, determinations were made on an unhemolyzed sample and on a totally hemolyzed sample and included in the average total radioiron (T_m) calculated below.

Calculation of tagged cell hemolysis was done as follows:—

Let R_s = radioiron in supernatant,
R_c = radioiron in unlysed cells,
T = radioiron in 0.5 ml. of blood,
H = per cent hemolysis of tagged cells,

then $R_{s1} + R_{c1} = T_1$ for any individual tube

and $R_{s_2} + R_{c_2} = T_2$, etc.

$\frac{T_1 + T_2 + T_3 \cdots}{n} = T_m$ = average radioiron in 0.5 ml. of blood.

and

$\frac{R_s}{T_m} \times 100 = H$, and $100 - \left(\frac{R_c}{T_m} \times 100\right) = H$. The average of the two values of H was computed.

After the above determinations had been completed the total circulating radioiron in the dogs' erythrocytes was readily calculated. This calculation was based on the assumption of a blood volume of 80 ml. per kilo body weight, and correction was made for the radioactivity removed in sampling. These figures are reported as per cent of administered dose.

EXPERIMENTAL OBSERVATIONS

Experiment 1.—Dog 48-113. Male mongrel. Weight 10.45 kilos. Approximately one-half of this dog's blood volume was removed by two venesections about 24 hours apart. Immediately after the second bleeding 0.87 mg. of radioactive iron was given intravenously.

The results of the measurements of osmotic fragility at various intervals are shown in Fig. 1. The curves are essentially the same as those reported by Cruz and his coworkers (6). The data for cells over 63 days of age are omitted to conserve space, but it can be stated that there was no detectable difference between the curve for the tagged cells and the curve for the total cell population. The curves obtained at 63 days were essentially like those at 120 days.

The results of the determinations of mechanical fragility of this dog's erythrocytes are shown in Fig. 2. The per cent hemolysis of the cell population as a whole should be compared with the per cent hemolysis of the tagged cells at each interval. It is apparent that the young cells are more resistant than the cells of average age and that the older cells become less resistant to the trauma of rolling glass beads.

The total circulating radioiron expressed as per cent of the injected dose is also shown in Fig. 2. It will be noted that the curve rises sharply for $4\frac{1}{2}$ days and then reaches a plateau. This curve reveals that the tagged cells are within about 2 days of a common age. "Sharp" tagging of this sort can be obtained only with a relatively small dose of radioactive iron which means that the counts per minute of some samples may be so low as to imperil the accuracy of the measurements of radioactivity. This difficulty was encountered in Experiment 1. The counts for the first 2 days were at the borderline of reliability in samples having a low hemoglobin concentration. The "mates" of the samples having low counts, however, had adequate radioactivity, thus rendering the data reliable even for the first 12 hours.

The total circulating radioactive iron remained at a rather constant level until the 98th day. At this point it declined precipitously until the 119th day, at which time it began to rise and was still rising at a slow rate when the last

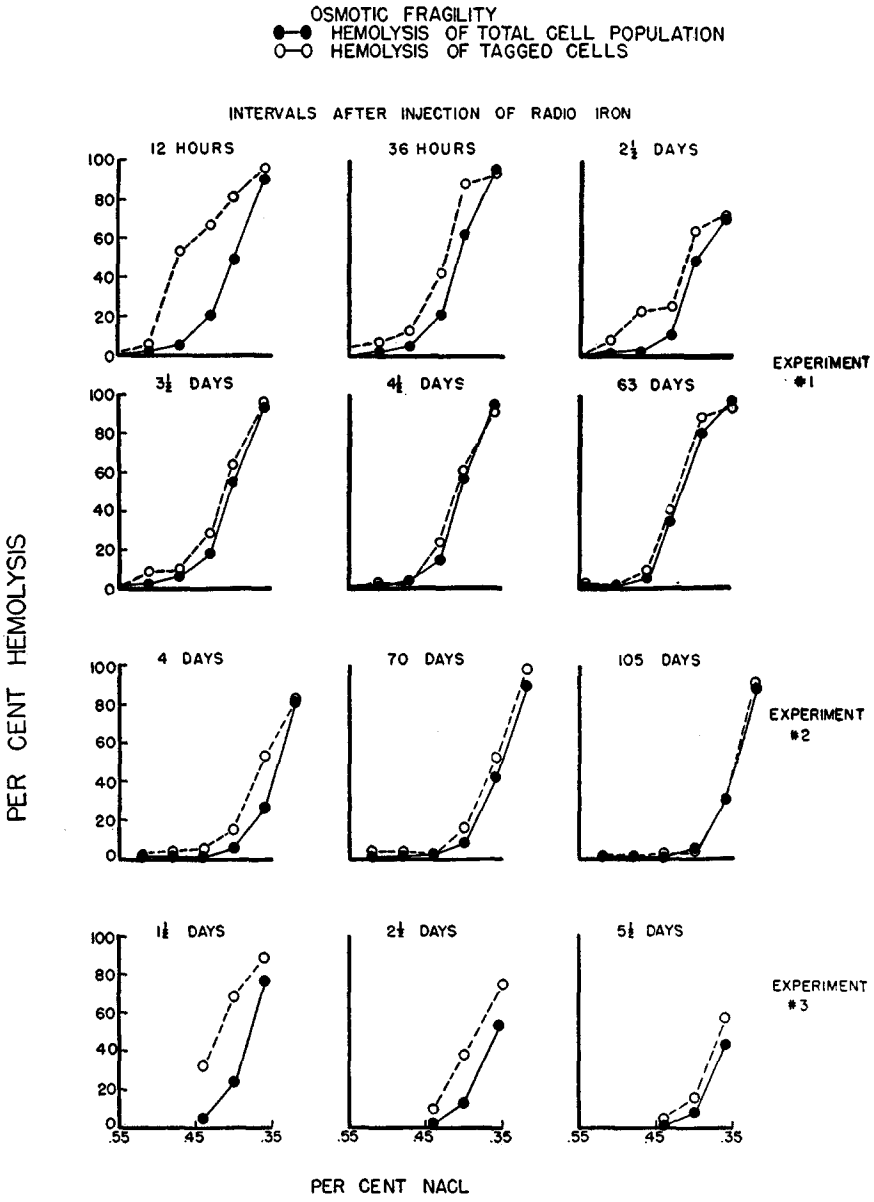

FIG. 1. Osmotic fragility of tagged dog erythrocytes and of total cell population at various intervals after injection of radioactive iron in Experiments 1, 2, and 3.

measurement was made on the 154th day. The assumption seems warranted that the dip in the curve for circulating radioiron is a reflection of the destruc-

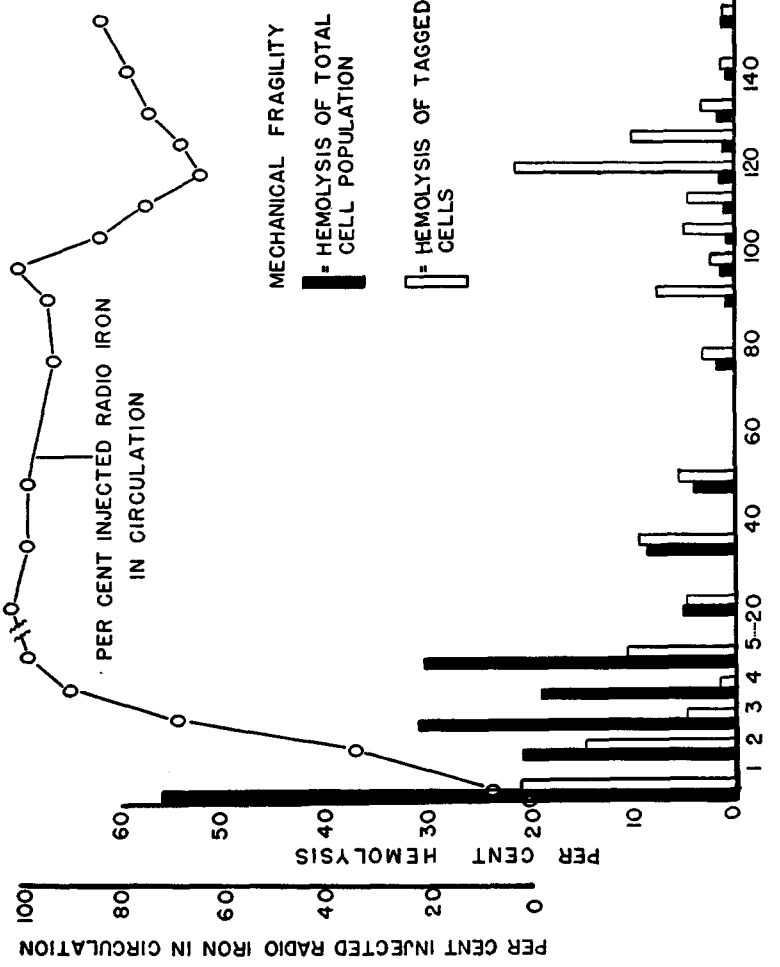

FIG. 2. Experiment 1. Mechanical fragility of tagged dog erythrocytes and of total cell population at various intervals after injection of radioactive iron. Circulating radioiron at each interval is expressed as per cent of dose injected.

tion of the obsolescent tagged cells, with temporary removal of the radioiron from circulation, soon followed by its reincorporation into newly formed erythrocytes. The curve as plotted would seem to be the algebraic sum of the curve of destruction of old cells and the curve of the reincorporation of the radioactive iron into new cells. If the low point of the curve is taken as a measure of the life span of the average red cell, a figure of 119 days is obtained, which is in good agreement with measurements of dog erythrocyte life span by bile pigment excretion (14, 23), selective agglutination of red cells (26), and by experiments employing radioactive carbon (1). This point does not yield a precise determination of the true life span, however, since the picture is confused by the reutilization of the radioactive iron.

The reutilization of radioactive iron must also be taken into account in interpreting the measurements of fragility. After a maximum difference at 119 days the mechanical fragility of the tagged cells and that of the total cell population approached equality while the radioactivity was being transferred to young erythrocytes. During this period and probably for several weeks earlier the activity must have been present in old cells as well as young cells. In view of this distribution, the changes observed from the 98th to the 153rd day are even more impressive.

Another factor entering into the matter is that bleeding results in the formation of a large group of new cells of nearly similar age. The normal distribution of cells with respect to age is thereby distorted, and in interpreting measurements of "differential" fragility it must be appreciated that the tagged cells comprise a considerable portion of the total cell population. Boulton (2) has suggested, moreover, that such cells produced under conditions of bone marrow stress are not of normal fragility. These considerations prompted the authors to carry out Experiment 2 which was performed without bleeding the dog prior to the injection of radioactive iron.

Experiment 2.—Dog 48-129. Male mongrel. Weight 15 kilos. This dog was given an intravenous injection of 30 mg. of radioactive iron. It was anticipated that, without previous bleeding of the animal, the per cent of utilization would be relatively low, and hence that the counts of radioactivity would also be low unless a large quantity was given. Under the circumstances described the circulating radioactive iron increased more slowly than in the first experiment and consequently the age distribution of the tagged cells was relatively wide.

The observed trends (Figs. 1 and 3) were the same as in the first experiment but the differences were less striking. The young cells were osmotically more fragile and mechanically more resistant. The maximum difference in mechanical fragility of old cells occurred at 105 days while the minimum of the curve of total circulating radioactivity was at 119 days. The less abrupt fall in circulating radioiron in this experiment is a reflection of the greater spread in the age of the tagged cells. The osmotic fragility of the old cells was again not detectably different from that of the cells of average age.

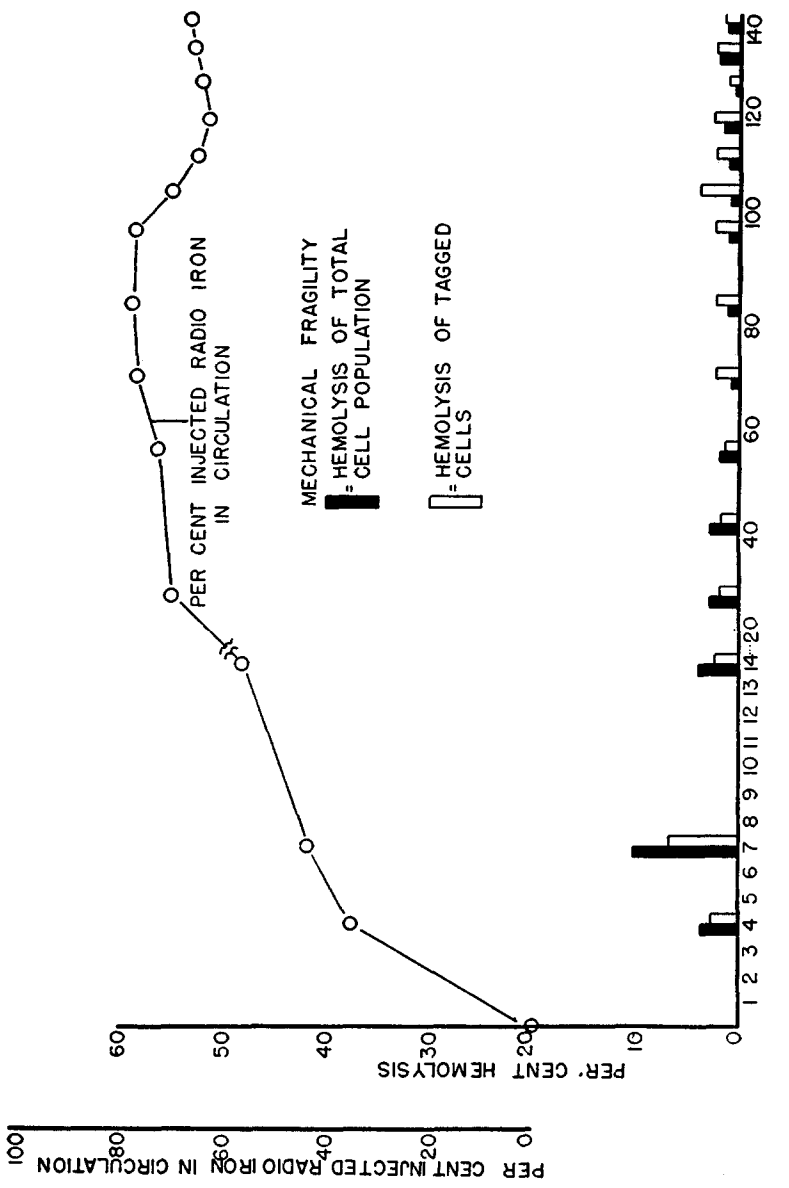

FIG. 3. Experiment 2. Circulating radioiron and mechanical fragility of dog erythrocytes plotted at various intervals after injection of radioactive iron.

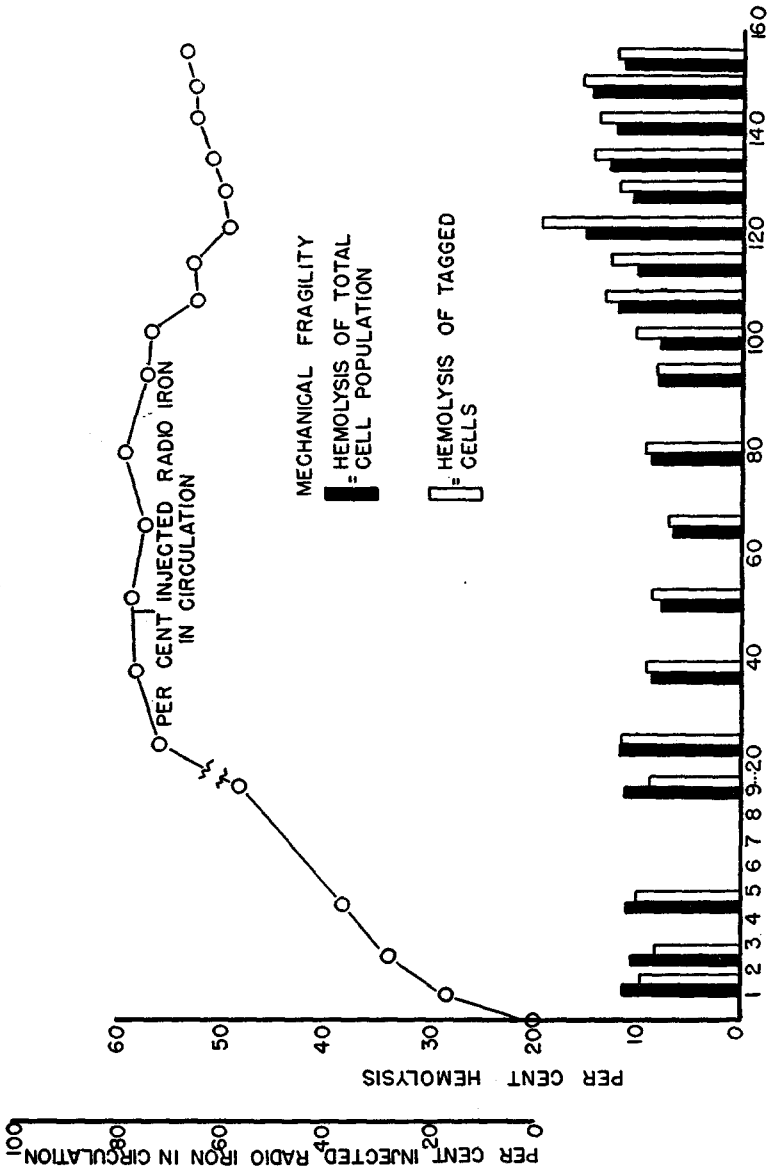

FIG. 4. Experiment 3. Circulating radioiron and mechanical fragility of dog erythrocytes at intervals after injection of radioactive iron.

The mechanical hemolysis of the total cell population in Experiments 1 and 2 was rather variable and in some instances in Experiment 2 was low enough to jeopardize the accuracy of the measurements of radioactivity. The variations in mechanical fragility of red cells observed in these experiments were, however, no greater than those frequently encountered in other studies on dog erythrocytes in this laboratory. The exact causes of the greater variation in mechanical hemolysis of dog cells as compared with human cells are unknown (21), but it should be emphasized that both the tagged and untagged cells were at all times subjected to exactly the same conditions. In the third experiment the procedure was modified as previously described in order to produce larger amounts of hemolysis (usually 8 to 15 per cent) at the time of each determination.

Experiment 3.—Dog 48-222. Spayed female mongrel. Weight 11.4 kilos. After two bleedings, which together removed about one-third of the blood volume, 3.65 mg. of radioactive iron was injected intravenously.

Data from this experiment, shown graphically in Figs. 1 and 4, again confirmed the earlier results. Determinations of osmotic hemolysis in this experiment were made only at 0.44, 0.40, and 0.36 per cent sodium chloride. The mechanical fragility of the total cell population, as determined by the modified technique, remained more nearly constant than in the previous experiments. The differences between the mechanical hemolysis of the tagged cells and that of the total cell population were not as great as in the other experiments but nevertheless followed the same pattern.

The curve of total circulating radioiron reached a plateau in about 21 days; thus, the age distribution of the tagged cells was rather wide. This curve, like those in the previous experiments, later showed a dip which reached its lowest point at 122 days.

DISCUSSION

The processes responsible for elimination of senescent erythrocytes at the end of their life span in normal animals have for some time been the subject of speculation. Ponder (18) lists four "extrinsic processes" which may result in red cell destruction *in vivo*: (1) osmotic hemolysis, (2) action of hemolysins, (3) mechanical destruction, and (4) phagocytosis. Hypotonicity sufficient to lyse red cells is not encountered in normal animals and can be produced only temporarily by intravenous injection of water or by use of large amounts of water for irrigation during surgical operations. The rôle of hemolysins (16), such as lysolecithin, in *in vivo* destruction of red cells has not been clearly established. Phagocytosis of either whole erythrocytes or red cell fragments by cells of the reticulo-endothelial system may be an important mechanism for disposal of effete cells, but quantitative studies of this phenomenon have not been reported.

Although the spleen plays a major rôle in bringing about hemolysis by one mechanism or another in certain hemolytic diseases, the amount of red cell destruction caused by the normal spleen is now thought to be small. Gordon and Kleinberg (8) and Singer and Weisz (23) have in fact found that the life span of the red cell is unaffected by splenectomy in the guinea pig and dog, respectively.

The experiments described in this paper reveal that newly formed dog erythrocytes are relatively resistant and old cells relatively susceptible to destruction by rolling glass beads. To our knowledge, there have been no previously recorded measurements of alterations in senescent cells that might be related to the events terminating the life of erythrocytes *in vivo*. It is reasonable to suggest that mechanical fragility of red cells, as measured rather crudely *in vitro*, may be an index of the susceptibility of cells to destruction by wear and tear in the circulation.

In order to produce measurable hemolysis of a sample of red cells *in vitro* within a relatively short period of time (90 minutes) the cells must be subjected to treatment much more harsh than that inflicted by the circulation. Ponder (18) has made the suggestion that it may be as difficult to buffet a red cell *in vivo* "as it would be to buffet a bag of feathers." Despite theoretical shortcomings of the mechanical fragility test, however, the results of these determinations have correlated well with measurements of the ability of various types of cells to survive *in vivo* (5, 12, 15, 22). The increased mechanical fragility of aging tagged cells may therefore be regarded as presumptive evidence that susceptibility to trauma is a factor limiting the life of erythrocytes. The relative importance of mechanical destruction of red cells in the circulation can be determined only by further study.

Evidence pointing to the rôle of mechanical factors in normal red cell destruction has also been described by Rous and Robertson (19, 20) who concluded that erythrocytes are destroyed by fragmentation in the circulation. Additional indirect evidence is found in the studies of Broun who observed reticulocytosis (4) and a decrease in total blood volume (3) in dogs exercised after a period of inactivity. McMaster, Broun, and Rous (17), moreover, found an increase in bilirubin excretion in bile fistula dogs exercised vigorously after being previously sedentary.

The results shown in Fig. 1 of this paper confirm those of Cruz, Hahn, Bale, and Balfour (6), who demonstrated that young erythrocytes tagged with radioactive iron are more susceptible to lysis in hypotonic saline than are red cells of average age. Evidence to the contrary has been described for the most part by observers using histologic methods for differentiation of young cells (6). It should be emphasized that the young cells tagged with radioactive iron are not necessarily reticulocytes, since red cells newly released from

the marrow may not always be reticulated (27). It should also be pointed out that the observations described in this paper were obtained with normal dogs, and that in certain pathologic states the osmotic fragility of young cells might actually be less than that of older cells. This might be particularly true in some of the human hemolytic disorders in which sphering is seen only in mature erythrocytes.

The cause of the increased osmotic fragility of newly formed dog cells is not clear. Experience with human red cells from patients with a variety of hematologic disorders has revealed a number of instances in which the mechanical fragility was increased while the osmotic fragility was normal or less than normal, but the reverse has rarely been observed (15, 22). It was therefore of especial interest that the osmotically fragile young dog cells actually showed less mechanical fragility than did cells of average age.

Failure to demonstrate a significant change in osmotic fragility of cells near the end of their life span is not difficult to understand. The differences may be too small to detect by the method used; the reincorporation of radioactive iron into young cells may obscure the differences, or there may be no difference in the osmotic fragility of old cells as compared with red cells of average age.

The curves representing circulating radioactive iron in Figs. 2, 3, and 4 deserve separate comment, since a dip was observed in each of these curves during the period when elimination of tagged cells from the circulation was presumably maximal. The lowest point of the dip was recorded at 119, 119, and 122 days respectively in the three experiments. These figures agree well with estimates of the life span of dog red cells provided by other methods. This unexpected finding has not been encountered in earlier studies employing radioactive iron (11) probably because: first, the low radioactivity of the iron administered; second, the large doses of iron employed; third, the greater spread in the age of the tagged cells; and fourth, infrequent sampling of the subject's blood. Finch *et al.* (7), however, have recently noted similar decrements in circulating radioactive iron in dogs and in human subjects under circumstances somewhat different from those obtaining in the experiments described in this paper.

SUMMARY

Radioactive iron was administered to three normal dogs, two of which had previously been bled, in order to tag a group of erythrocytes of approximately known age.

The osmotic fragility of the newly formed tagged cells was significantly greater than that of the general cell population during the first few days after injection of the iron, while the mechanical fragility of the young cells was less than that of the general red cell population. As the cells aged and approached

the end of their life span, their susceptibility to destruction by trauma inflicted by rolling glass beads exceeded that of the general cell population. The osmotic behavior of the old cells was not distinctive.

The increased mechanical fragility of senescent cells suggests that the life span of erythrocytes may be limited at least in part by changes within the cell which render it more susceptible to destruction by mechanical wear and tear in the circulation. It is emphasized, however, that the trauma produced by rolling glass beads may be quite unlike that inflicted upon red cells *in vivo*.

A decrease in circulating radioactive iron was observed in each experiment soon after the mechanical fragility of the tagged cells began to exceed that of the total cell population. The lowest point on the curve representing circulating radioiron was noted at 119, 119, and 122 days respectively after injection of iron in the three experiments. Estimates of the life span of dog erythrocytes obtained in this way agree with those provided by other methods.

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