Endogenous Production of $^{14}$CO: A Method for Calculation of RBC Life-span In Vivo

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A kinetic model is presented for estimation of the degradation rate of labeled heme by measurement of appearance of $^{14}$CO in the breath following injection of glycine-$^{14}$C. This method does not require sampling of blood or other body fluids, is absolutely independent of circulating blood volume and relatively independent of erythropoietic rate, and estimates the relative contribution to $^{14}$CO production from destruction of circulating RBC hemoglobin heme and that arising from other heme sources. For circulating RBC, rate of random hemolysis, mean potential lifespan and spread of lifespans about this mean can be calculated. Mean overall RBC lifespan and the fraction of RBC dying of senescence can be derived from these calculations. In normal male buffalo rats, the average value for random hemolysis was 0.67 per cent per day, corrected mean potential lifespan 66.2 days and standard deviation about this mean of 7.6 days. For normal female LAF$_1$ mice, the corresponding average values were 0.60, 51.8 and 9.1, respectively. Results in two other inbred mouse strains were similar save for a shorter mean potential lifespan of 47.1 days in SEC/1Re mice and a longer mean potential lifespan of 57.3 days in WC-B6 mice. Following splenectomy in the rat, an isolated significant increase in mean potential lifespan was seen. An isolated decrease in mean potential lifespan was seen in three rats recovering from pherylhydrazine-induced anemia. An example of markedly increased random hemolysis, together with shortened mean potential life-span, was seen in a gastrectomized rat with a severe hypochromic anemia. The present method is shown to simultaneously determine the major parameters defining RBC survival, and as such, should be quite useful in the study of red blood cell disorders in animals and man.

TRACER METHODS previously available for the determination of the survival of circulating red blood cells have been adequately reviewed elsewhere, and generally require the collection of multiple blood samples following injection of suitably labeled materials. Recently, methods were introduced for the in vivo detection and quantitation of endogenously produced $^{14}$CO in the breath following the injection of glycine-$^{14}$C, the metabolic source for the four methene bridge carbon atoms of heme. When
such labeled heme is catabolized, the heme ring is opened at the alpha-methene bridge carbon atom, with the quantitative oxidation of this carbon atom to $^{14}$CO. The $^{14}$CO thus produced is excreted rapidly and quantitatively in the breath without significant oxidation to $^{14}$CO$_2$. Therefore, the excretion rate of $^{14}$CO in the breath reflects the destruction rate of labeled heme in the body, allowing heme catabolism to be studied in intact animals without venesection or collection of other body fluids. In this paper, a kinetic analysis of RBC destruction will be presented using analysis of $^{14}$CO excretion in mice and rats having normal RBC survival patterns and also in animals with representative disorders affecting RBC survival.

**Materials and Methods**

*General Procedure*

Animals used were 300-400-Gm. male specific pathogen-free buffalo rats (Simonsen Laboratory, Gilroy, Calif.), and 17-26-Gm. female LAF$_1$, SEC/1Re, and WC-B6 mice (courtesy of Dr. E.S. Russell and Dr. S.E. Bernstein, Jackson Laboratory, Bar Harbor, Maine). Rats were studied individually following intravenous injection of 50 $\mu$Ci of glycine-$2^{14}$C (specific activity: 20-28 mCi/mM, New England Nuclear Corp., Boston, Mass.) under light ether anesthesia. Each experiment in mice was performed in groups of from two to five animals, each simultaneously injected intravenously with 10 $\mu$Ci of labeled glycine.

Labeled carbon monoxide ($^{14}$CO) was detected and quantitated by a method previously described. This method entails the removal of contaminating $^{14}$CO$_2$ from the expired air by means of soda lime, oxidation of the $^{14}$CO to $^{14}$CO$_2$ by means of a Hopcalite catalyst (Mine Safety Appliances Co., Pittsburgh, Pa.) and absorption of the $^{14}$CO$_2$ thus generated in an ethanolamine-containing solution, followed by liquid scintillation counting. Previous studies have shown that this continuous in vivo method is an accurate method for measuring degradation of labeled heme(s) in intact animals, and that contamination by endogenously-produced $^{14}$CO$_2$ and other volatile compounds in breath, urine and feces is negligible.

*Blood Studies*

Uptake of labeled glycine into heme and hemoglobin of circulating RBC was studied in normal buffalo rats and LAF$_1$ mice. Small samples of peripheral blood (less than 0.15 ml. each) were removed via tail vein puncture of rats at various times after injection of labeled glycine; the total amount of blood removed from each rat was less than 5 per cent of the calculated blood volume. Red cells were washed three times in saline and hemin was extracted and crystallized from these small samples of blood by a direct micromodification of the method of Labbe and Nishida. Hemin concentrations were determined spectrophotometrically and activity was determined by liquid scintillation.

Uptake of glycine into RBC hemoglobin was studied by plating samples of stroma-free RBC hemolysates on aluminum planchettes and counting with a gas-flow proportional counter. Uptake of the $^{14}$carbon into hemoglobin and heme was analyzed using visual or computer best fit to Equation 4 (see Appendix).

*Preparation of Experimental Animals*

*Cross-transfusion studies.* A group of normal rat donors was injected intravenously with 50-100 $\mu$Ci of glycine-$2^{14}$C and sacrificed either 24 hours or 14 days later. Blood was removed by aortic puncture and washed three times in isotonic saline. Hematocrits were adjusted to 45-55 per cent by the addition of saline and 2-3 ml. were injected intravenously into normal rat hosts.

*Hypertransfusion studies.* Female LAF$_1$ mice were hypertransfused by intraperitoneal
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injection of packed RBC from isologous donors. Each mouse received 1 ml. of packed RBC (hematocrit 60–70%) intraperitoneally 29, 28 and 3 days prior to injection of labeled glycine. At the latter time, the animals' hematocrits were in the range of 60–70 per cent. Ten μCi of glycine-2-14C were then injected into each mouse intravenously (day zero). Hematocrits were maintained in the range of 55 per cent or above by further injections of packed RBC on the 22nd, 39th and 44th days after labeled glycine injection. The rate of erythropoiesis was assessed in these animals by measurement of the incorporation of isotope into circulating RBC 72 hours after the injection of tracer doses of 59Fe.

Hypochromic anemia following gastrectomy. A male Sprague-Dawley rat developed hypochromic anemia shortly after total gastrectomy.* The anemia responded completely to parenteral iron dextran therapy, but over the next 12 months, the animal's blood picture slowly reverted to the original picture of a severe hypochromic anemia. The animal was studied when the anemia had stabilized at a hemoglobin level of approximately 3 Gm. per cent.

Phenylhydrazine-induced anemia. Three rats were given 4 mg. of phenylhydrazine per 100 Gm. of body weight per day subcutaneously on days 0, 1 and 2. One rat was then injected with 50 μCi of labeled glycine 3, 7 or 11 days following the third dose of phenylhydrazine.

Splenectomy. A male buffalo rat was injected with 50 μCi of labeled glycine 39 days after splenectomy, at which time the hematocrit and reticulocyte counts had returned to preoperative levels.

Hypophysectomy. Two male buffalo rats were injected with 50 μCi of labeled glycine 106 days following transoral hypophysectomy. Due to an inadequate intake of food, these animals were unable to maintain a stable weight and died approximately 75 days after injection of labeled glycine.

Data Analysis

All data for 14CO production obtained more than 3 days after glycine injection were fitted to appropriate mathematical formulae (see Appendix) with a variable metric minimization ('VARMIT') least-squares fitting program.13 This is an iterative gradient method which determines local minima of differentiable functions. The program, as modified by E. R. Beals, Lawrence Radiation Laboratory, Berkeley, was run on a Control Data CDC-6600 digital computer. Copies of this program are available on request. The results of RBC survival obtained from such programs are stated in terms of the following parameters:

- \( k \), rate of random hemolysis (per cent per day)
- \( T \), mean potential RBC lifespan (days)
- \( \sigma \), spread of lifespans about the mean potential lifespan (days)
- \( C \), fraction of injected glycine incorporated into RBC heme (%)
- \( S \), fraction of labeled RBC dying of senescence (%)
- \( T \), mean overall RBC lifespan (days)

In addition, the contribution to 14CO production from the catabolism of nonhemoglobin hemes and that due to the persistence of label in the glycine pool was computed for each animal by assuming that these processes could be described by the sum of two or more exponential terms (see Appendix).14 In these calculations it was assumed that the turnover rate of the slower exponential process was identical in all animals within a given species; the slope of this exponential term (\( k_2 \)) was fixed at the value determined in hypertransfused mice for all mouse studies and at the value determined in the starved hypophysectomized rats for all rat studies. The amplitude of this exponential term (\( A_2 \)) was allowed to vary in the fitting function, except in those cases in which insufficient data was available for time periods greater than \((T) + 2(\sigma)\). In this case the amplitude was determined by the visually estimated exponential function which the data appeared to approach asymptotically.

*Kindly supplied by Dr. Samuel Lepkovsky, Department of Poultry Husbandry, University of California, Berkeley, Calif.
Fig. 1.—Endogenous 14CO excretion rate in groups of normal and hypertransfused LAF1 mice. This graph shows a semilogarithmic plot of 14CO excretion rate (ordinate, dpm/hour of 14CO) versus time after injection of glycine-2-14C (abscissa, days) in a group of five normal LAF1 mice (open circles) and a group of four hypertransfused LAF1 mice (closed circles). The solid lines represent the least squares best fit of the data points to equation 8. The half-times for the two exponential components are given on the figure.

RESULTS

Hypertransfused Mice

Fig. 1 demonstrates 14CO appearance in the breath of mice in the presence and absence of erythropoiesis. In the latter case, RBC production was completely suppressed by hypertransfusion (closed circles), as evidenced by the complete disappearance of the "late peak" seen in the controls (open circles). In addition, 72-hour uptake of 59Fe into circulating RBC was 0.3-0.6 per cent in the hypertransfused mice, as compared to 18-38 per cent in the controls. Production of 14CO by the hypertransfused mice 3-110 days after injection of labeled glycine could be approximated by the sum of two exponential terms, with half-times of 2.7 and 52 days. Two similar terms could be noted in the 14CO curve of the controls.

Hypophysectomized Rats

Curves of 14CO excretion in two starved hypophysectomized rats in which erythropoiesis was greatly depressed could be approximated by the sum of two exponential terms, as for the hypertransfused mice, with half-times of 2.5 and 100 days (Fig. 2). No distinct "late peak" was seen in one rat, while a minor peak with an absolute magnitude not more than 4 per cent of normal was seen in the other (Fig. 2, open circles).

Cross-Transfusion Studies

In cross-transfused rats, a definite "late peak" for 14CO was noted (Fig. 3, Table 1), with a maximum at about 63 days. There was no evidence for the
slower exponential process seen in the normal or hypertransfused mice (Fig. 1) or in the starved hypophysectomized rats (Fig. 2).

**Normal LAF₁ Mice and Buffalo Rats**

Figures 4A and B present the rate of ¹⁴CO excretion in a normal buffalo rat and a group of five normal LAF₁ mice, respectively. These curves define the time at which the maximum rate of ¹⁴CO excretion is seen (late peak) as approximately 65 days in the rat and 53 days in the mouse. An early rapid exponential process and a late slow exponential process are both seen in these figures, in addition to the late peaks. Parameters of RBC survival determined from such curves are summarized in Tables 1 and 2 for buffalo rats and three different inbred mouse strains. Results for all parameters were essentially identical in the three mouse strains except for the mean potential lifespan (T) which varied from 47 days in the SEC/1Re strain to 57 days in the WC-B6 strain. The appearance of the ¹⁴carbon label in the heme of circulating RBC hemoglobin was studied in three rats and in a group of simultaneously injected LAF₁ mice and yielded values for the mean labeling time of circulating RBC hemoglobin heme (1/λ) of 0.9 days for the rat and 1.1 days in the mouse.

**Phenylhydrazine-Induced Anemia**

The three animals with phenylhydrazine-induced anemia showed marked increases in ¹⁴CO excretion from 3 to 9 times normal (Table 1), with a well-defined late peak centered about 50 (Fig. 4C), 56 and 62 days, respectively, in contrast to the range in the controls of 65 to 68 days (Table 1). The general
shape of the $^{14}$CO curves did not otherwise appear to be appreciably altered from that of the normal rats.

**Hypochromic Anemia Following Gastrectomy**

In the gastrectomized rat (Fig. 4D), the virtual absence of the late peak is noted, the remnants of which can be appreciated as a shoulder in the curve at 40 to 50 days after isotope injection. The $^{14}$CO curve may be approximated by a single exponential function between 7 and 40 days following isotope injection, with a half-time of 10 days, corresponding to a rate of random hemolysis of 7 per cent per day, or about 10 times that seen in normal rats (Table 1).

**Splenectomized Rat**

In the splenectomized rat, the only alteration from normal in $^{14}$CO production was a late peak centered about 72 days (Fig. 4E), approximately 6 days longer than that noted in normal rats (Table 1).

**Discussion**

Experiments in the hypertransfused mice and starved hypophysectomized rats demonstrate that, following the third day after glycine-2-$^{14}$C injection, nonerythropoietic components of endogenous $^{14}$CO production can be approximated by the sum of two exponential terms. The first of these, with a half-time of 2.06 ± 0.25 (SE) days in all the mice and rats herein reported, is identified at least in part with heme compounds having rapid turnover such as has been postulated by others.16-21 The slower of these two components, with a half-time of 52 days in the mouse and 100 days in the rat, probably represents a
### Table 1.—Results of Standard Hematologic Tests Plus Parameters of RBC Survival in Normal and Experimental Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Average or Range of Six Normal Buffalo Rats (Mean ± SE)</th>
<th>Average of Six Cross-Transfused Male Buffalo Rats (Fig. 3)</th>
<th>Anemia Due to Phenylhydrazine (Fig. 4C)</th>
<th>Hypochromic Anemia Following Gastrectomy (Fig. 4D)</th>
<th>Buffalo Rat Postsplenectomy (Fig. 4E)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standard Hematologic Tests</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin (Gm. %)</td>
<td>13.5-14.7 Range</td>
<td>—</td>
<td>31.5 43.0 48.0</td>
<td>12.9 44.7</td>
<td>14.2</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>44-50 Range</td>
<td>—</td>
<td>— 30.1 56.0* 61.7*</td>
<td>44.41 71.8*</td>
<td></td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin concentration (Gm. %)</td>
<td>29-32 Range</td>
<td>—</td>
<td>— 30.1 56.0* 61.7*</td>
<td>44.41 71.8*</td>
<td></td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>1.5-3.5 Range</td>
<td>—</td>
<td>80 64 20</td>
<td>32</td>
<td>2.3</td>
</tr>
<tr>
<td><strong>Parameters of RBC Survival:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rate of random hemolysis (%/day) (k)</td>
<td>0.67 ± 0.07</td>
<td>0.60 ± 0.05</td>
<td>0.79 0.38 0.35</td>
<td>7.02† 0.52</td>
<td></td>
</tr>
<tr>
<td>Corrected mean potential life-span (days) (T)</td>
<td>66.2 ± 0.7</td>
<td>63.0 ± 0.7</td>
<td>50.1 56.0* 61.7*</td>
<td>44.41 71.8*</td>
<td></td>
</tr>
<tr>
<td>Standard deviation about mean potential life-span (days) (σ)</td>
<td>7.6 ± 0.6</td>
<td>8.6 ± 0.4</td>
<td>8.1 7.1 9.4</td>
<td>5.6 9.3</td>
<td></td>
</tr>
<tr>
<td>Per cent uptake of glycine into RBC heme (%) (C)</td>
<td>0.247 ± 0.032</td>
<td>—</td>
<td>2.230† 1.444† 0.781‡</td>
<td>0.517* 0.331</td>
<td></td>
</tr>
<tr>
<td>Mean overall RBC life-span (days) (T)</td>
<td>54.5 ± 1.5</td>
<td>53.3 ± 0.6</td>
<td>42.2* 51.3 56.2</td>
<td>14.51 60.6</td>
<td></td>
</tr>
<tr>
<td>Per cent of RBC death by senescence (%) (S)</td>
<td>64.8 ± 2.7</td>
<td>69.0 ± 2.7</td>
<td>67.6 81.1 80.5</td>
<td>4.91 68.8</td>
<td></td>
</tr>
</tbody>
</table>

* *p < 0.05, parameter in italics
† † † p < 0.001, parameter in italics
Table 2.—Parameters of RBC Survival in Three Strains of Normal Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LAF1</th>
<th>SEClReJ</th>
<th>WC/B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Animals Studied</td>
<td>15</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Rate of random hemolysis (per cent per day) ((k))</td>
<td>0.60 ± 0.17*</td>
<td>0.32</td>
<td>0.54</td>
</tr>
<tr>
<td>Corrected mean potential lifespan (days) ( (T'))</td>
<td>51.8 ± 0.4</td>
<td>47.1</td>
<td>57.3</td>
</tr>
<tr>
<td>Standard deviation about mean potential lifespan (days) ((\sigma))</td>
<td>9.1 ± 1.2</td>
<td>8.8</td>
<td>7.8</td>
</tr>
<tr>
<td>Per cent uptake of glycine into RBC heme (per cent) ((C))</td>
<td>0.323 ± 0.003</td>
<td>0.299</td>
<td>0.411</td>
</tr>
<tr>
<td>Mean overall RBC lifespan (days) ((\bar{T}))</td>
<td>45.6 ± 1.6</td>
<td>44.8</td>
<td>50.3</td>
</tr>
<tr>
<td>Per cent of RBC death by senescence (per cent) ((S))</td>
<td>74.1 ± 6.7</td>
<td>86.2</td>
<td>73.5</td>
</tr>
</tbody>
</table>

* Mean ± SE.

number of processes, including turnover of glycine-containing proteins which release their glycine into the free glycine pool from whence a portion is reincorporated into rapidly catabolized tissue heme compounds.

Since in a number of studies there were not sufficient data points to define precisely the slope of the slowest exponential, data from mice were pooled and yielded a half-time of 52 days, which was used in fitting all mouse data. Similarly, a half-time of 100 days was found suitable for all rat data, including that obtained in hypophysectomized rats. This was taken then as the value used in all subsequent rat data analysis. If it is assumed that each data point has no inherent variability other than the fixed sources of error such as pipetting and counting errors, the computer program was able to calculate the precision of each determined parameter. The average coefficient of variation for the values herein reported were \((\%)\): \((k)\) 4.06; \((T)\) 0.28; \((\sigma)\) 2.23; \((C)\) 1.72; \((A_1)\) 5.03; and \((k_1)\) 3.00. Possible variations in the slope and amplitude of the slow exponential process did not significantly affect the values for \((T), (\sigma), (\bar{T})\) or \((S)\) but did introduce an uncertainty in \((k)\) which did not exceed 0.20 per cent per day. However, the average value of 0.67 per cent per day for \((k)\) in six normal male buffalo rats did not differ significantly \((p > 0.50)\) from the average value obtained in six cross-transfused rats (0.60 per cent per day) in which the slow exponential process is absent. As the erythropoietic rate decreases, the magnitude of the late peak relative to the slow exponential process decreases, thus decreasing the precision of calculations for \((k), (\sigma), (C)\), \((A_1)\), and \((k_1)\). While the value for \((T)\) can probably always be determined graphically with good precision. At erythropoietic rates less than 25 per cent of normal, the net late peak signal is equal in value to the inherent variability in the data points. Thus, in the presence of markedly reduced erythropoietic rate (with normal RBC survival), the accuracy of the \(^{14}\)CO method diminishes.

Belcher and Harriss reported values for \((k)\) of 0.22–0.69 per cent per day in rats,\(^{24}\) in good agreement with values herein reported. Their values for the spread of life-spans about the mean potential life-span (calculated values of \((\sigma)\) of 3.7–8.5 days) are also in agreement with results from the \(^{14}\)CO method. In general, reported values for the mean potential lifespan \((T)\) using the \(^{59}\)Fe method (59–62 days\(^{24,26}\)) are less than those obtained with the hemin-\(^{14}\)C technique (68 days).\(^{27}\) Studies performed in this laboratory in male buffalo
Fig. 4.—Endogenous 14CO excretion under various experimental conditions. Ordinate and abscissa same as in Fig. 1. Data points represented as solid circles. The solid line represents the least squares best fit of Equation 8 to the data points (accounting for all sources of 14CO). The finely dashed line represents the calculated production of 14CO from the destruction of the cohort of labeled RBC (Equation 2), while the coarsely dashed line represents the calculated production of 14CO from the destruction of labeled nonhemoglobin hemes as well as recycling of labeled glycine. (A) Normal buffalo rat; (B) a group of five normal, simultaneously injected LAF1 mice; (C) buffalo rat recovering from phenylhydrazine-induced anemia; (D) Sprague-Dawley rat with hypochromic anemia postgastrectomy; and (E) splenectomized buffalo rat.
rats using the $^{59}\Fe$ method also confirmed a value for $(T')$ of 59.6 days, 6 days shorter than the value obtained in the same rat strain using the $^{14}\CO$ method (Table 1). This shortened survival probably reflects toxic alterations due to multiple injections of iron dextran.

The average value for the fractional incorporation of labeled glycine into circulating hemoglobin heme $(C)$ herein reported from $^{14}\CO$ analysis was lower than results obtained in different animals in this study from direct measurement of glycine incorporation into RBC heme $(0.403\% \pm 0.039\% \text{ SE})$ and with those reported by Robinson et al. in the Gunn rat $(0.62 \pm 0.23\%)$. The basis for this difference cannot be established from retrospective analysis of the data, but the possibility of consistent technical errors in early experiments is suggested by the observation that the most recent $^{14}\CO$ experiments yielded values of $(C)$ which were not significantly different from values obtained from direct circulating hemoglobin activity measurements, for example, in the normal rat depicted in $(C)$. These results may be explained by postulating either residual "damage" to labeled cells by phenylhydrazine, or that the "quality" of such cells is somehow progressively impaired with increasing RBC production rate, leading to earlier senescence. Evaluation of such mechanisms will be the subject of a subsequent publication. In four of the normal rats injected with washed glycine-labeled RBC, the total amount of $^{14}\CO$ recovered in the breath over the first 100 days ranged from 90.5 to 101.1 per cent (average, 94.8\%) of the activity present in the alphamethylene bridge carbon atoms of the injected heme. Thus, when heme activity in the cohort is directly compared to the total recovery of $^{14}\CO$, agreement is excellent. Reproducibility of this method is attested to by the closeness between results herein reported and results obtained at a separate laboratory using this method in rats obtained from the same supplier: $(k) 0.69; (T') 68.3; (\sigma) 6.4; (C) 0.265; (\bar{T}) 55.3$, and $(S) 62.5$.

An example of isolated shortening in the mean potential life-span $(T)$ was seen in rats recovering from phenylhydrazine-induced anemia (Fig. 4C and Table 1). In these three rats, the mean potential life-span became more normal as the hematocrit, reticulocyte count, hemoglobin, and fractional glycine incorporation returned to normal. These results, which have been confirmed in other rats given similar treatment, differ from those previously reported by Stohlman in which the phase of senescence was not specifically studied but which were reported to show increased rates of random hemolysis. In these and similarly treated rats, the shortening of the mean potential lifespan was directly proportional to the value obtained for $(C)$. These results may be explained by postulating either residual damage to labeled cells by phenylhydrazine, or that the quality of such cells is somehow progressively impaired with increasing RBC production rate, leading to earlier senescence. Evaluation of such mechanisms will be the subject of a subsequent publication.

An example of markedly increased random hemolysis and marked shortening of mean potential life-span was seen in the rat with postgastrectomy hypochromic anemia (Fig. 4D, Table 1). Shortened RBC survival in iron deficiency in animals and man has been reported; one report indicates a short-
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ening of mean potential life-span\textsuperscript{32} and another can be interpreted as evidence of an increased rate of random hemolysis.\textsuperscript{34} Further studies of animals made iron deficient by the usual dietary means must be performed before the abnormalities described above can be considered typical of severe iron deficiency.

An example of an isolated increase in the mean potential lifespan following splenectomy is shown in Fig. 4E. The mean potential lifespan of 72 days (Table 1) was significantly ($p < 0.05$) greater than in more than 70 rats with normal or abnormal erythropoiesis studied by this method; the resulting increase in mean overall life-span to about 61 days (normal 54.5 ± 1.5 SE days) was not statistically significant; however ($p > 0.10$). Belcher and Harriss\textsuperscript{35} had also reported that splenectomy increased the measured value of ($T$) from 60 days in the normal to 65-69 days.

Red blood cell survival in the cross-transfused rats (Fig. 3, Table 1) did not differ significantly from any of the measured parameters in normal rats except for a 4.5 per cent shortening of ($T$) from 66 days to 63 days ($p < 0.05$). Such shortening of ($T$) following cross-transfusion has been regularly noted in both normal and phenylhydrazine-treated cells and very likely represents trauma imposed on the RBC from handling, washing and injection procedures.

Previous determinations of RBC survival in mice have yielded conflicting results, with one study reporting that RBC death was due solely to random processes,\textsuperscript{35} while others suggest the complete absence of random hemolysis.\textsuperscript{36-38} The present study indicates that the rate of random hemolysis in LAF\textsubscript{1} mice is approximately equal to that seen in the rat, with a mean potential lifespan of about 52 days (Table 2). Values for ($T$) of 47 and 57 days in the SEC/1Re and WC-B6 strains are significantly different from that found in the LAF\textsubscript{1} mice, suggesting that some of the previously reported variations in ($T$) in the mouse\textsuperscript{36-39} may be due to genetically determined variations.

Figure 5 demonstrates that the present $^{14}$CO method can reproduce the shape of the expected RBC hemoglobin or heme activity curves. In this figure, the shaded area represents the range of hemin-$^{14}$C activity curves expected in rats from Equation 1 (Appendix), using parameters derived from $^{14}$CO breath data, exclusive of variations in the size of such cohorts (C, see above). Data points were obtained from the blood of rats in which the specific activity of hemoglobin or heme was serially followed. These data points are entirely consistent with the parameters derived from the $^{14}$CO method. However, the great advantages of this method are that it avoids the unphysiologic effects of multiple blood sampling, allows RBC survival to be determined accurately in a single animal despite rapidly changing blood volume and/or erythropoietic rate and can be applied easily to animals breathing artificial atmospheres without removing them from their environment.\textsuperscript{30} Recently, Coburn\textsuperscript{40} and White et al.\textsuperscript{17} have shown that similar late peak curves can be obtained in man, suggesting that this technique is also applicable in man.

ACKNOWLEDGMENT

The authors are indebted to Dr. John H. Lawrence for supporting all phases of this work and to Mr. Mark Horowitz and Mrs. Sandra Bristol for valuable technical assistance.
Fig. 5.—Comparison of measured circulating RBC heme $^{14}$C activity curves with those calculated from $^{14}$CO excretion in six normal buffalo rats. The ordinate represents normalized circulating RBC heme $^{14}$C specific activity (normalized to 100, see text for discussion of possible errors in amplitude (C)), while the abscissa represents days after labeled glycine injection. The shaded area represents the range of circulating RBC hemoglobin heme activity curves calculated from $^{14}$CO excretion rate data and Equation 1. The different symbols each represent measured heme specific activity curves in a single normal buffalo rat. (Closed triangles represent a curve derived from hemoglobin specific activity in a single rat.)

Determination of the mean RBC labeling time in LAF1 mice was performed by Mr. Norman Zucker during a Summer Fellowship sponsored by the U. S. Atomic Energy Commission.

APPENDIX

Mathematical treatment of heme activity curves has been adequately presented elsewhere. The simplest formula applied to senescent and random destruction of a cohort of labeled RBC is derived from the Verhulst-Pearl growth curve and has the form:

$$F(t) = \frac{C e^{-kt}}{1 + e^{(a)(t-T)}}$$  \hspace{1cm} (1)

where $F(t)$ is the activity of hemoglobin heme at a time $t$ following appearance of tracer in the circulating RBC, $C$ is the maximum activity attained, $T$ is the mean potential life-span, $(k)$ the rate of random hemolysis and $(a)$ the coefficient of uniformity of life-spans about $(T)$. This model, as well as its application to $^{14}$CO kinetics, assumes that these parameters are invariant through the life of the cohort of labeled RBC. Since the rate of production of $^{14}$CO equals one-eighth of the destruction rate of labeled heme, the production rate of $^{14}$CO is given by one-eighth of the first derivative with respect to time of the heme activity curve, denoted by $F'(t)$ or $dF/dt$. This is shown in Equation 2, where the minus sign is introduced since the activity in $^{14}$CO increases as the activity in heme decreases:

$$-F'(t) = \frac{Ce^{-kt} (k + (k+a) e^{a(t-T)})}{8(1 + e^{a(t-T)})^2}$$  \hspace{1cm} (2)

Both the term $(a)$ of equations 1 and 2 and the $(a)$ term of the gaussian function are measures of the distribution of values about a mean. $(a)$, the standard deviation, increases as the spread about a mean increases, while $(a)$ decreases as the spread about a mean increases. When the sum of the squares of the differences between the gaussian and the
first derivative of the Verhulst-Pearl growth curve (Equation 2 with \( k = 0 \)) was minimized, the following relationship between \( a \) and \( \sigma \) was obtained:
\[
(a) \times (\sigma) \cong 1.701
\]  
(3)

Allowance for delayed appearance of the labeled cells into the circulation is made by studying the early phase of glycine incorporation into circulating RBC. As was shown by Shemin and Rittenberg, this phase can be approximated by:
\[
H(t) = C (1 - e^{-t})
\]
(4)

where \( H(t) \) is the activity of heme in circulating RBC. The noninstantaneous appearance of label in the circulating RBC causes an error in the determination of the true mean potential RBC lifespan \((T')\) on the order of the mean labeling time \((1/\lambda)\) as follows:
\[
T' \cong T - 1/\lambda
\]
(5)

The average survival time for circulating RBC \((\bar{T})\) for both random hemolysis and senescence was determined from:
\[
(\bar{T}) = \frac{8}{C} \int_{0}^{\infty} (t) \times F'(t) \, dt
\]
and the per cent of cells destroyed by random hemolysis \((RH)\) from:
\[
(RH) = \frac{800}{C} \int_{0}^{\infty} (k) \times F(t) \, dt
\]
and
\[
(S) = 100 - (RH),
\]
where \( S \) is the per cent of circulating RBC dying of senescent processes.

Labeled CO may arise from degradation of hemes other than that in hemoglobin (e.g., heme enzymes). Furthermore, following the initial passage of labeled glycine through the glycine pool, the labeled glycine may recirculate, resulting in the labeling of hemes synthesized subsequent to the initial cohort.22 The \(^{14}\) CO arising from these processes can be empirically described by a sum of exponential terms (Figs. 1 and 2). In practice, only two terms were required to account for such processes.14 Since uncertainties in the slower exponential term can influence the derived parameters of RBC survival, this model assumes that those processes influencing this term do not change significantly throughout the experiment (nonhemoglobin heme turnover, rate of recirculation of glycine, rate of erythropoiesis, etc.). The following equation is thus fitted to the \(^{14}\) CO data using a least-squares fitting program, and the resulting parameters \((C), (k), (T), (a)\) were used to describe RBC survival and to calculate the derived parameters \((T'), (\bar{T}), (RH), (S), (a)\):
\[
CO(t) = \frac{Ce^{-kt} (k+k+a)e^{\alpha(t-\bar{T})}}{8(1 + e^{\alpha(t-\bar{T})})^2} + A_1e^{-k_1t} + A_2e^{-k_2t}
\]
(8) 

(for \( t \geq 3 \))

REFERENCES


CALCULATION OF BBC LIFE-SPAN

Endogenous Production of $^{14}$CO: A Method for Calculation of RBC Life-span In Vivo

STEPHEN A. LANDAW and H. SAUL WINCHELL