The Use of ^{14}C-Cyanate for Red Blood Cell Survival Studies (37099)

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Cerami and Manning (1) and Gillette, Manning and Cerami (2) have shown that cyanate reacts *in vivo* and *in vitro* in a carbamylation reaction with the amino terminal end of hemoglobin. The irreversible nature of this chemical bonding suggested to them that cyanate should make an excellent label for RBC survival studies (3), without elution characteristic of chromium labeling (4). The present study was undertaken to determine the suitability of ^{14}C-cyanate as a random RBC label in the rat.

Materials and Methods. Animals used were male buffalo rats, weighing 350–500 g (Simonsen Laboratory, Gilroy, CA). Potassium-^{14}C-cyanate (sp act 6 mCi/mmole, New England Nuclear, Boston, MA) was incubated directly with heparinized rat whole blood in a concentration of 7–10 μCi/ml, at 37° for 30–60 min. Cells were then washed up to 3 times in phosphate-buffered saline, and 1 ml injected intravenously into host rats. In a separate series, ^{14}C-cyanate was dissolved in saline, and 10 μCi were injected iv into each of 3 host animals.

Blood was obtained 24 hr after injection, and every 1–2 wk thereafter by tail vein puncture, under light ether anesthesia. Not more than 0.25 ml was obtained at any one time, and the total volume removed did not exceed 6% of estimated blood volume for the entire experiment. Blood was collected directly into heparinized microhematocrit tubes, and centrifuged for 2–2.5 min to delineate the buffy coat. The tube was broken just below the buffy coat layer and at its lower end with a diamond point, RBC were transferred to saline, washed 3 times, and finally suspended in approximately 200 μl of isotonic saline. Fifty microliter aliquots were used for determination of hemoglobin concentration, using a standard cyanmethemoglobin method. Similar 50 μl aliquots were pipetted in duplicate into counting vials containing 0.5 ml of a saturated solution of benzoyl peroxide in toluene. Three milliliters of solubilizer (NCS Solubilizer, Nuclear-Chicago, Des Plaines, IL) were then added, followed by incubation at 50° for 30 min to facilitate complete solubilization (5). Fifteen milliliters of scintillator solution were then added (PPO, 5.5 g/liter of toluene), and the homogeneous, slightly yellow solution was dark adapted for at least 3 hr before counting by liquid scintillation, after optimization of operating parameters to exclude the low energy scintillations characteristic of chemiluminescence. Counting efficiency was determined for each sample using a ^{14}C-toluene internal standard; efficiency (36–41%) decreased only slightly with increasing amounts of hemoglobin in the range from 1.6 to 9.6 mg. Background count rates (10–14 cpm) were identical for colorless (saline blank) or colored (non-labeled RBC) solutions. Initial count rates of host RBC at 24 hr were 20–80 times background; duplicate samples always agreed to within 2%.

The curve of hemoglobin specific activity (dpm/mg hemoglobin) versus time after labeling was fitted for each group of 3 animals to the following function, modified from Eadie and Brown (6), using a least-squares method and a digital computer:

$$H(t) = C\,(1 - R/T)\,e^{-kt}, \qquad (1)$$

where:

$$R = t - \frac{\ln\,(1 + e^{a(t-T)})}{a}.$$

This formula describes the specific activity of RBC t days after labeling, when cells are destroyed by random hemolysis k and senescence (mean potential lifespan T, and coefficient of uniformity of lifespans a).

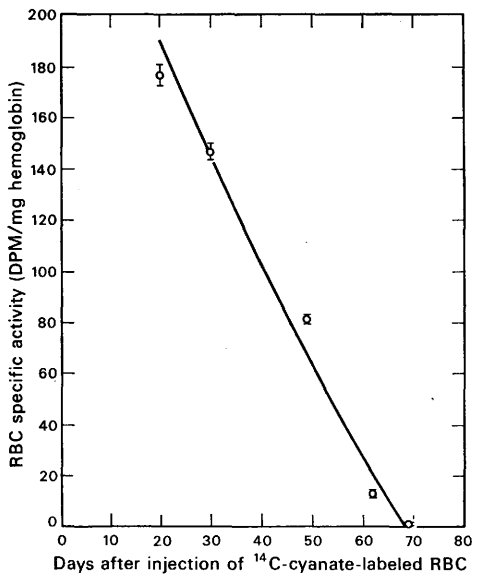

FIG. 1. Survival curve in Expt. 1. Each data point describes RBC hemoglobin specific activity (mean ± SE) for a group of 3 rats, at various times following injection of RBC labeled *in vitro* with ^{14}C-cyanate. (—) Least-squares best fit of data points to Eq. (1).

Initial values of T were obtained by the method of Eadie and Brown (6); mean overall lifespan $\overline{T}$, and the standard deviation of lifespans about the mean potential lifespan (sigma) were determined as previously described (7). Coefficients of variation for the initially determined parameters averaged 25% for (sigma), 5% for k and less than 1 % for T.

Initial data points were fitted to a linear regression line using a least-squares method. The reciprocal of the slope of this line is equal to the mean overall lifespan $\overline{T}$ (8).

Results and Discussion. After 60 min of incubation of 8 ml of RBC with 80 μCi of ^{14}C-cyanate at 37°, hemoglobin specific activity was 1.71×10^4 dpm/mg. After buffy coat removal and triple washing, the 1.02 g hemoglobin in the resulting RBC suspension contained 7.8 μCi of activity, equal to an *in vitro* incorporation of ^{14}C-cyanate into RBC of 9.8%. Twenty-four hours after injection of 1 ml of this RBC suspension (1.12 μCi), hemoglobin sp act in 5 hosts averaged 605 dpm/mg. Assuming a blood volume of 5% body weight and a hemoglobin concentration of 17 g/100 ml, this amounted to a total circulating activity of 1.06 μCi. Thus, 95 ± 3 (SE)% of initially injected activity was present in the circulation 24 hrs postinjection.

In 3 animals given 10 μCi of ^{14}C-cyanate iv, RBC hemoglobin sp act averaged 156 dpm/mg 24 hr postinjection, equivalent to an incorporation of 2.80 ± 0.17 (SE)% of the iv dose into circulating RBC. Cerami (9) has recently reported an incorporation of from 5 to 10% of administered cyanate into hemoglobin of experimental animals.

In 2 samples of postinjection peripheral blood, the specific activity of washed, WBC-free RBC (145 and 152 dpm/mg Hb) was equal to that of a stroma-free saponin-induced hemolysate prepared from the same RBC suspension (146 and 153 dpm/mg), indicating that no appreciable activity was associated with the RBC membrane or other formed elements in the blood.

Activity in circulating RBC was barely detectable (extinction time) 66–73 days after *in vivo* or *in vitro* labeling (Figs. 1–3). The extinction time was in all cases longer than the mean potential lifespan T due to cells surviving longer than T, inherent in the distribution of cell death about T (6–8). Such

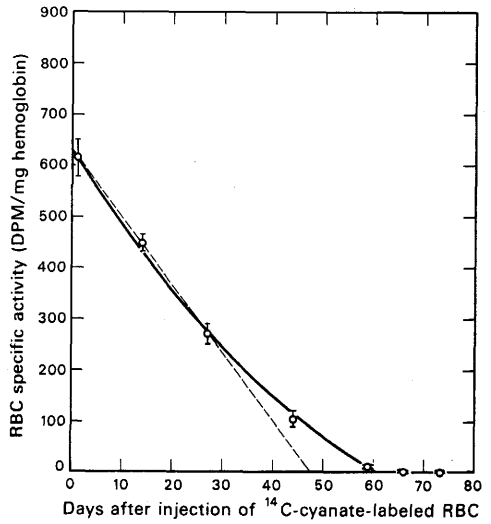

FIG. 2. Survival curve in Expt. 2. Data points and solid line as for Fig. 1, for a group of 3 rats injected with RBC labeled *in vitro* with ^{14}C-cyanate. (– –) Initial linear segment of survival curve.

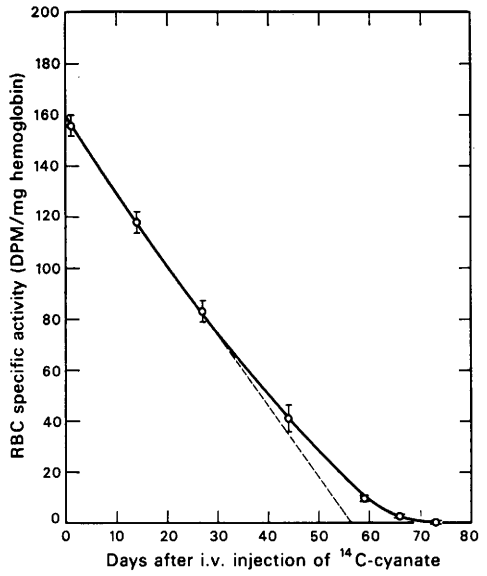

FIG. 3. Survival curve in Expt. 3. Points, solid line, and broken line as for Fig. 2, for a group of 3 rats injected with ^{14}C-cyanate intravenously.

lifespan distribution affects Eq. (1) only for t greater than 40 days in the rat, at a time when absolute count rates were quite low; the precision of the calculated value for (sigma) is thus low for these random label experiments. Mean potential lifespan T, on the other hand, could be determined with good precision from Eq. (1). The values obtained in Expts 1 and 3 (68.2 and 64.0 days) are in close agreement with that obtained from cohort studies [66.2 $\pm$ 0.7 days (7)]. The shorter value in Expt 2 (60.6 days) was probably a result of the increased RBC trauma from longer incubation and repeated cell washing (*vide infra*).

Initial data points obtained 1–27 days after labeling were linear, with an initial slope indicating a mean overall lifespan $\overline{T}$ of 47.6 days following injection of *in vitro* labeled RBC (Expt 2, Fig. 2), and 56.4 days following direct iv injection (Expt 3, Fig. 3). Internal consistency was shown by the excellent agreement noted for $\overline{T}$ when calculated from the initial slope and from the best-fit parameters from Eq. (1) (Table I). Results from Expts 1 and 3 are in close agreement with the value of 54.5 $\pm$ 1.5 (SE) days obtained for $\overline{T}$ in cohort studies from this laboratory, using rats of identical strain and weight range (7). A shorter mean *overall* lifespan $\overline{T}$ due to an acceleration of senescence (decreased mean *potential* lifespan T) has been noted in cohort studies for transfused, washed RBC (7). The most likely explanation for the shortening of survival noted in Expt 2 is the trauma incurred during the collection, washing, and reinjection of RBC. DeFuria, Graziano and Cerami (3) noted a similar shortening in lifespan of RBC labeled *in vitro* with ^{14}C-cyanate in an anemic basenji dog, compared to the lifespan obtained after iv labeling. Although this handling of RBC may also lead to some immediate destruction, such irreversibly damaged RBC appear to be cleared rapidly from the circulation (10); the high recovery of initially injected activity at 24 hr argues against significant loss of RBC via this route.

The rate of random hemolysis k noted in

TABLE I. Comparison of RBC Survival Parameters from ^{14}C-Cyanate and Cohort Labels.

Expt and fig. no.	^{14}C-Cyanate labeling	Random hemolysis (k) (%/day)	Mean potential lifespan (T) (days)	Distribution of lifespans about T (sigma) (days)	Mean overall lifespan ($\overline{T}$) (days)	
					From initial slope	From eq. (1)
1	*In vitro*[a]	0.46	68.2	9.0	—	58.5
2	*In vitro*[b]	0.90	60.6	7.0	47.6	47.0
3	*In vivo*	0.42	64.0	6.0	56.4	56.3
Cohort study	—	0.67 $\pm$ 0.07[c]	66.2 $\pm$ 0.7	7.6 $\pm$ 0.6	—	54.5 $\pm$ 1.5

[a] Incubation 30 min at 37°. RBC not washed after labeling.
[b] Incubation 60 min at 37°. RBC washed 3 times after labeling.
[c] Mean $\pm$ SE for a group of 6 male buffalo rats [Ref. (7)].

the 3 groups ranged from 0.42 to 0.90%/day, in good agreement with cohort data (Table I). Since any significant amount of label elution would increase the apparent value for *k*, this indicates that long-term elution of ^{14}C-cyanate from RBC hemoglobin is negligible. This behavior is similar to that noted with diisopropylfluorophosphate (DFP), and in contrast to chromium, in which elution rates are in excess of 1%/day in both animals and man (4, 11, 12). The findings noted in the present study are consistent with preliminary reports on the lack of adverse hematologic effects of large doses of cyanate (1, 9) in animals, and evidence that RBC from patients with sickle cell disease incubated with cyanate *in vitro* show a markedly improved survival when reinjected into the original donors (2, 13). Such studies suggest that cyanate remains bound for the lifetime of the cell, and will not compromise its lifespan, major therapeutic goals in the treatment of sickle cell disease.

Summary. When incubated with rat RBC *in vitro,* or following direct intravenous injection, ^{14}C-cyanate is firmly bound to RBC of all ages. Red blood cell lifespan can be determined by following circulating RBC hemoglobin specific activity, yielding a value of 56–58 days for the mean lifespan, in agreement with that obtained from cohort studies. Similarly, mean potential lifespan (time of senescent death) and rate of random hemolysis were similar to that noted after cohort labeling. It is concluded that ^{14}C-cyanate is an excellent noneluting random label for RBC survival studies.

The author gratefully acknowledges a timely conversation with Dr. Frank A. Oski, which prompted the present study. This work was supported, in part, by a Career Development Award (5K4-HL-17693-03), from the National Heart and Lung Institute, National Institutes of Health.

1. Cerami, A., and Manning, J. M., Proc. Nat. Acad. Sci. USA **68**, 1180 (1971).

2. Gillette, P. N., Manning, J. M., and Cerami, A., Proc. Nat. Acad. Sci. USA **68**, 2791 (1971).

3. DeFuria, F. G., Graziano, J. H., and Cerami, A., J. Clin. Invest. **51**, 24a (1972).

4. Ebaugh, F. G., Emerson, C. P., and Ross, J. A., J. Clin. Invest. **32**, 1260 (1953).

5. Hansen, D. L., and Bush, E. T., Anal. Biochem. **18**, 320 (1967).

6. Eadie, G. S., and Brown, I. W., Blood **8**, 1110 (1953).

7. Landaw, S. A., and Winchell, H. S., Blood **36**, 642 (1970).

8. Dornhorst, A. C., Blood **6**, 1284 (1951).

9. Cerami, A., N. Engl. J. Med. **287**, 807 (1972).

10. Landaw, S. A., Blood **40**, 257 (1972).

11. Hughes Jones, N. C., and Cheney, B., Clin. Sci. **20**, 323 (1961).

12. Mollison, P. L., Clin. Sci. **21**, 21 (1961).

13. May, A., Bellingham, A. J., Huehns, E. R., and Beaven, G. H., Lancet **1**, 658 (1972).

Received Oct. 6, 1972. P.S.E.B.M., 1973, Vol. 142.