Title
The Use of 14-C-Cyanate for Red Blood Cell Survival Studies

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THE USE OF ^{14}C-CYANATE FOR RED BLOOD CELL SURVIVAL STUDIES

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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 non-eluting random label for RBC survival studies.

INTRODUCTION

Cerami and co-workers (1,2) 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 grams (Simonsen Laboratory, Gilroy, Calif.). Potassium-^{14}C-cyanate (specific activity 6 mCi/mM, New England Nuclear, Boston, Mass.) was incubated directly with heparinized rat whole blood in a concentration of 7-10 μCi/ml, at 37°C for 30-60 minutes. Cells were then washed up to 3 times in phosphate-buffered saline, and 1 ml injected intravenously into host rats. In a separate series,

DISCLAIMER

^{14}C-cyanate was dissolved in saline, and 10 μCi injected IV into each of 3 host animals.

Blood was obtained 24 hours after injection, and every 1-2 weeks thereafter by tail vein puncture, under light ether anesthesia. Not more than 0.25 ml was obtained at any one time, and the total removed volume 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 1/2 minutes 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 transferred to saline, washed 3 times, and finally suspended in approximately 200 microliters of isotonic saline. Fifty microliter aliquots were used for determination of hemoglobin concentration, using a standard cyanmethemoglobin method. Similar 50 microliter aliquots were pipetted in duplicate into counting vials containing 0.5 ml of a saturated solution of benzoyl peroxide in toluene. Three ml of solubilizer (NCS Solubilizer, Nuclear-Chicago, Des Plaines, Ill.) were then added, followed by incubation at 50°C for 30 minutes to facilitate complete solubilization (5). Fifteen ml of scintillator solution were then added (PPO, 5.5 grams per liter of toluene), and the homogeneous, slightly yellow solution was dark adapted for at least 3 hours 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 - 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 hours 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)). 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 minutes of incubation; approximately 1.4% of ^{14}C-cyanate was attached to the washed RBC. Assuming a blood volume

of 5% of body weight, and a hemoglobin concentration of 17 grams %, 95 ± 3 (SE) % of injected activity was in the circulation of host rats 24 hours after injection. In 3 animals given ^{14}C-cyanate IV, incorporation averaged 2.80 ± 0.17 (SE) % at 24 hours. In 2 samples, the specific activity of washed, WBC-free RBC (145 and 152 DPM/mg Hgb) 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 (Figures 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 lifespan distribution affects Equation (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 Equation (1). The values obtained in Experiments 1 and 3 (68.2 and 64.0 days) are in close agreement with that obtained from cohort studies (66.2 ± 0.7 days, (7)). The shorter value in Experiment #2 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 ($\bar{T}$) of 47.6 days following injection of in vitro labeled RBC (Experiment #2, Figure 2), and 56.4 days following direct IV injec-

tion (Experiment #3, Figure 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 Equation (1) (Table I). Results from Experiments 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 due to a decreased mean potential lifespan had been suggested in the cohort study for transfused, washed RBC; the most likely explanation for the shortening noted in Experiment 2 is the trauma incurred during the collection, washing, and re-injection of RBC. Similar results have been reported for ^{14}C-cyanate labeling of RBC in an anemic Basenji dog (3).

The rate of random hemolysis noted in the 3 groups ranged from 0.42 to 0.90 percent per day, in good agreement with cohort data (Table I). Together with the recovery of virtually all of initially injected activity (vide supra), this indicates that elution of the label from RBC does not occur to any great extent, similar to di-isopropylfluorophosphate (DFP), and in contrast to chromium, in which elution rates are in excess of 1% per day in both animals and man (4,9,10). 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) in animals, and evidence that RBC from patients with sickle cell disease incubated with cyanate *in vitro* retain this cyanate *in vivo* for long periods of time (2). 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.

ACKNOWLEDGEMENTS

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EXPERIMENT AND FIGURE #	^{14}C-CYANATE LABELING	(k) RANDOM HEMOLYSIS (PERCENT/DAY)	(T) MEAN POTENTIAL LIFESPAN (DAYS)	(SIGMA) DISTRIBUTION OF LIFESPANS ABOUT (T) (DAYS)	($\bar{T}$) MEAN OVERALL LIFESPAN (DAYS)	
					FROM INITIAL SLOPE	FROM EQUATION (1)
1	in vitro*	0.46	68.2	9.0	---	58.5
2	in vitro**	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 #	66.2 $\pm$ 0.7	7.6 $\pm$ 0.6	---	54.5 $\pm$ 1.5

*Incubation 30 minutes at 37°C. RBC not washed after labeling.

**Incubation 60 minutes at 37°C. RBC washed 3 times after labeling.

#Mean $\pm$ S.E. for a group of 6 male buffalo rats (Reference: (7)).

TABLE I

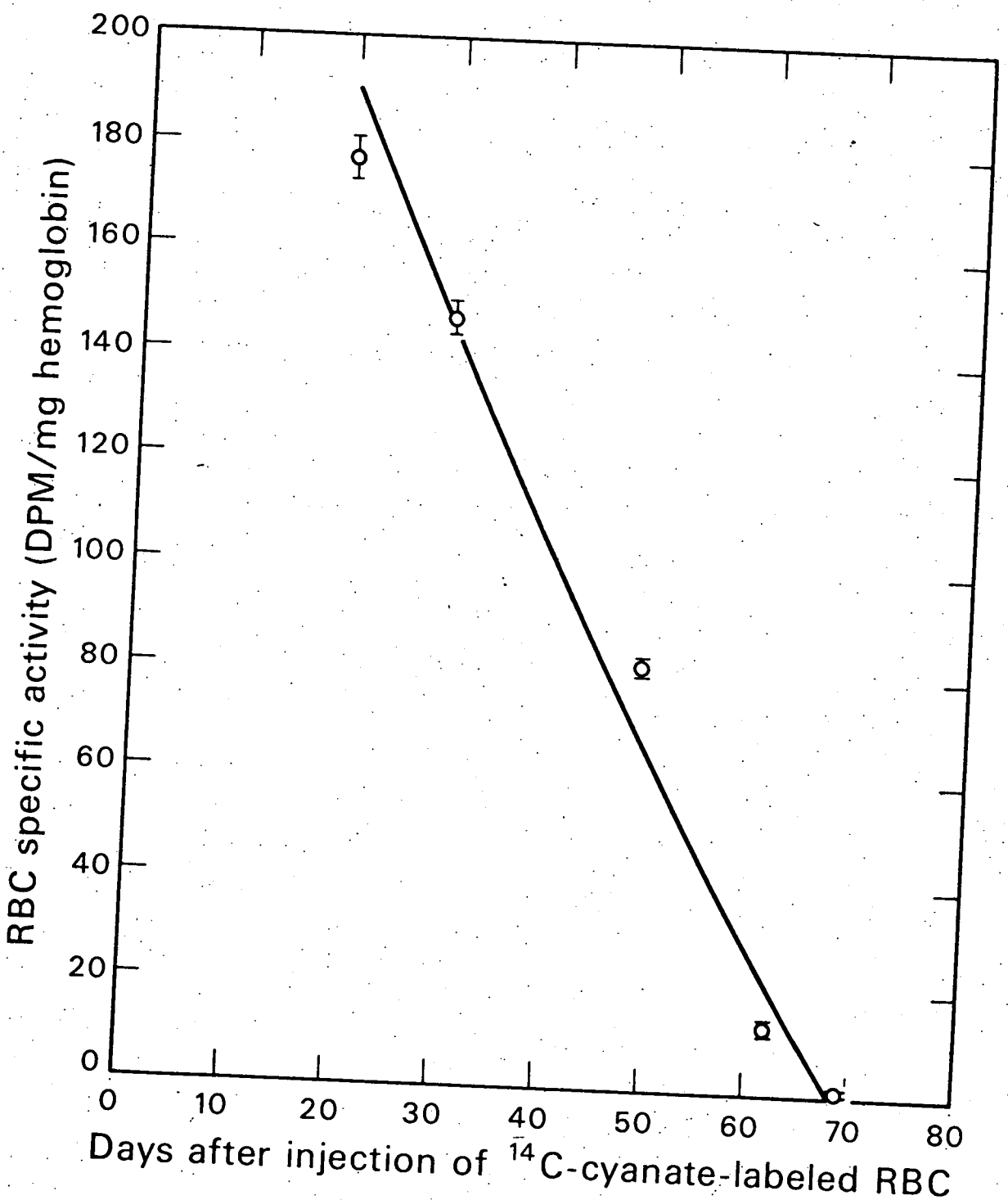
COMPARISON OF RBC SURVIVAL PARAMETERS FROM ^{14}C-CYANATE AND COHORT LABELS

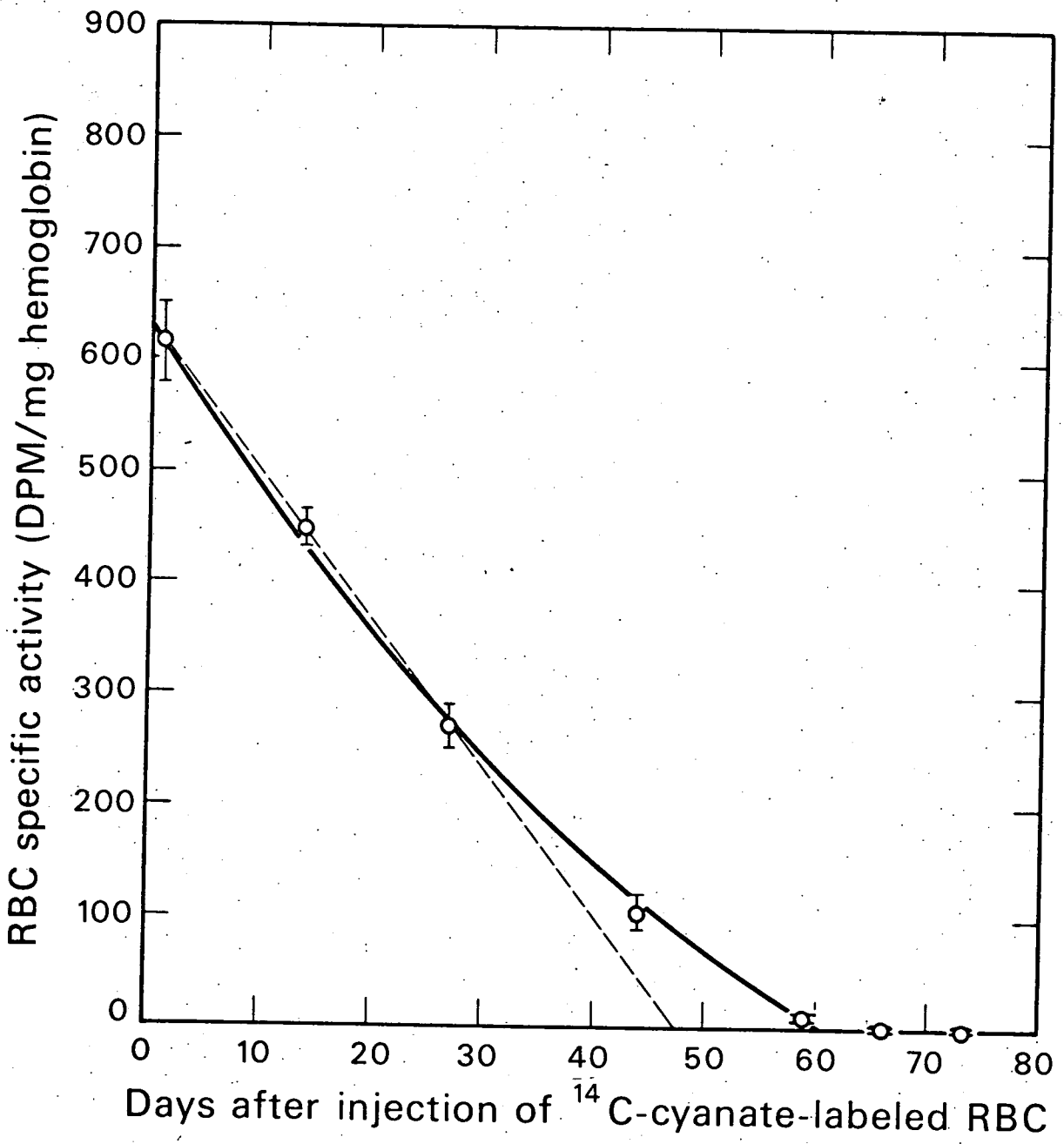
Figure Legends

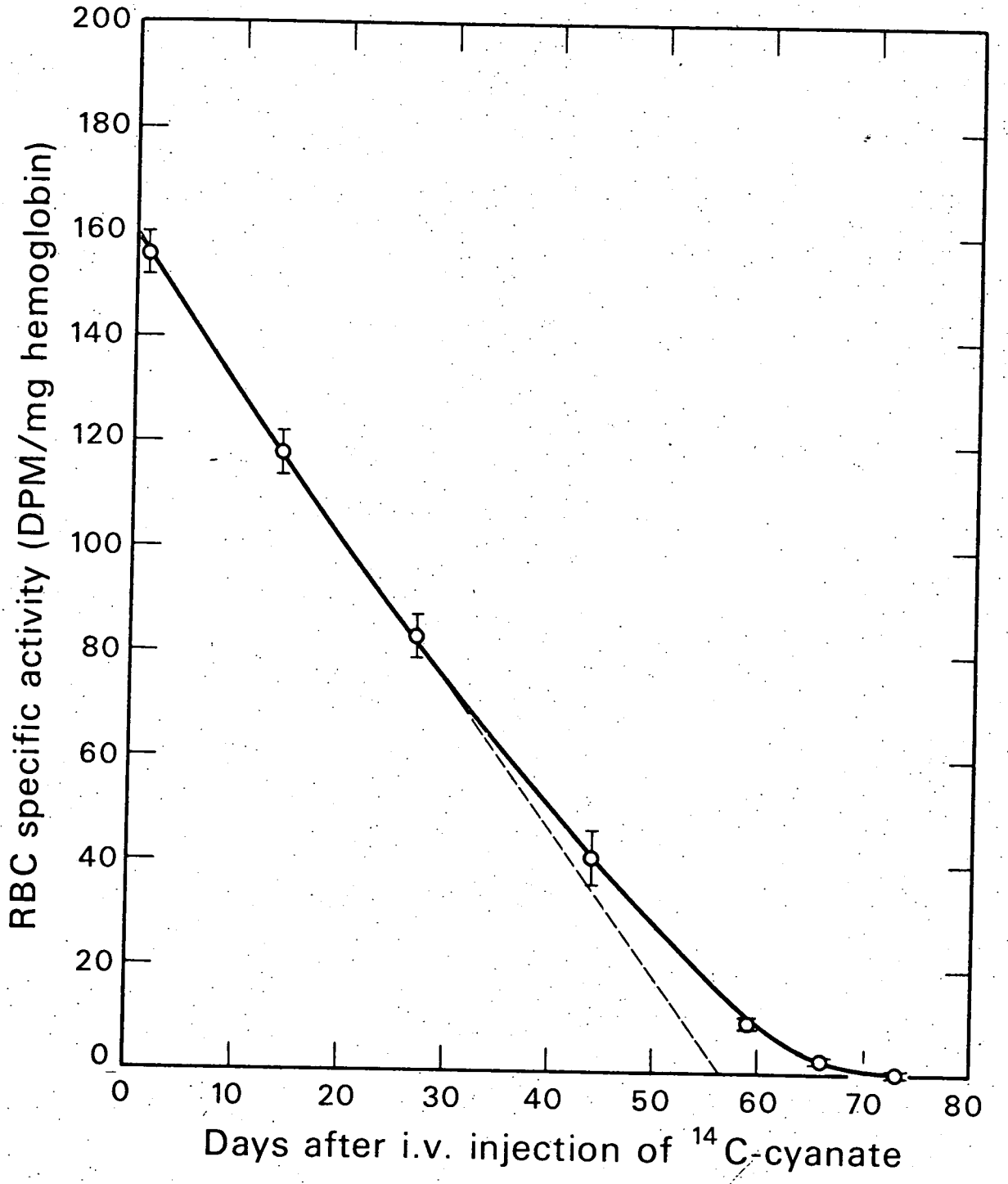
Figure 1: Survival curve in Experiment #1. Each data point describes mean ± S.E. for a group of 3 rats, following injection of RBC labeled *in vitro* with ^{14}C-cyanate. Solid line indicates least-squares best fit of data points to Equation (1).

Figure 2: Survival curve in Experiment #2. Data points and solid line as for Figure 1, for a group of 3 rats injected with RBC labeled *in vitro* with ^{14}C-cyanate. Dotted line indicates initial linear segment of survival curve.

Figure 3: Survival curve in Experiment #3. Points, solid line, and dotted line as for Figure 2, for a group of 3 rats injected with ^{14}C-cyanate intravenously.


RBC specific activity (DPM/mg hemoglobin)
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Days after injection of ^{14}C-cyanate-labeled RBC


900
800
700
600
500
400
300
200
100
0
RBC specific activity (DPM/mg hemoglobin)
0
10
20
30
40
50
60
70
80
Days after injection of ^{14}C-cyanate-labeled RBC


RBC specific activity (DPM/mg hemoglobin)
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Days after i.v. injection of ^{14}C-cyanate

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