The Use of ^{14}C-Cyanate as a Method for Determining Erythrocyte Survival[1] (37583)

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The estimation of erythrocyte survival is important in the evaluation of many anemias. Over the past 20 yr several radiolabeling techniques using ^{51}Cr and ^{3}H or ^{32}P-diisopropylfluorophosphate (DFP) have been developed for the determination of autologous erythrocyte survival (1, 2). These methods, although widely used, are not totally adequate since the loss of radioactivity from the blood cannot be directly equated with cell replacement. The elution of the reversibly bound ^{51}Cr and the hydrolysis of the triester-phosphate bond formed with DFP have led to difficulties in assessing erythrocyte survival time *in vivo*.

Cyanate has recently been found to react specifically with the amino-terminal valine of hemoglobin (3). This reaction is irreversible and has minimal effect on red cell metabolism and function (4). Consequently, we have developed the use of ^{14}C-cyanate as a method for determining red cell survival.

Methods. Animals: Four adult female rhesus monkeys (8 kg), five adult female beagle dogs (8 kg), and one basenji dog (5 kg) with a hemolytic anemia (hematocrit = 18) due to hereditary red cell pyruvate kinase deficiency (5) were studied.

Labeling with ^{51}Cr and ^{14}C-cyanate: Erythrocyte survival studies were done by a procedure modified from Gray and Sterling (6). Twenty-five milliliters of heparinized blood were drawn under sterile conditions, mixed with 5 ml of acid–citrate–dextrose solution (ACD; Squibb) and divided into two equal aliquots; 25 μCi of $Na_2{}^{51}CrO_4$ (Chromitope; Squibb; sp act, 30.4 Ci/g) were added to one aliquot and 25 μCi of $KN^{14}CO$ (New England Nuclear; sp act, 80 mCi/g) were added to the other.

The ^{51}Cr and ^{14}C aliquots were incubated at 37° for 60 min, the cells were washed three times with sterile saline, recombined and adjusted to a hematocrit of 30–40. A 3 ml sample was retained for hematocrit, hemoglobin, ^{51}Cr and ^{14}C determinations and the remainder was infused intravenously. Blood samples for hematocrit, hemoglobin, ^{51}Cr and ^{14}C determinations were taken at 30 min, at 1, 2, 4 and 7 days and weekly or biweekly for 6 to 8 wk. The sample taken 24 hr after reinfusion is considered to be Day 0 for both methods. In the present communication the apparent $T_{1/2}$ value for each of the methods is defined as the day at which one half of the radioactivity present 24 hr after infusion of the ^{51}Cr or the ^{14}C-cyanate labeled cells had been lost.

Triplicate 0.5 ml whole blood samples were stored in dialysis bags at +5°. They were subsequently dried under a heat lamp and oxidized in a Packard Tri-Carb sample oxidizer, which collects the evolved $^{14}CO_2$ in ethanolamine. The amount of radioactivity was determined in a Packard Tri-Carb liquid scintillation counter. The separation of the globin from the heme by an acid–acetone extraction (7) and the subsequent preparation of the globin for scintillation counting could be an alternative means of counting the ^{14}C radioactivity. One milliliter whole blood samples were stored at −5° and counted for

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^{51}Cr in a Nuclear Chicago gamma counter.

Two beagle dogs and the basenji dog with pyruvate kinase deficiency were injected intravenously with 5 ml of Millipore (0.45 μm) sterilized saline containing 100 μCi of $KN^{14}CO$. Blood samples were taken twice daily on Days 1, 2 and 4 and biweekly for several weeks. Since cyanate *in vivo* forms carbon dioxide (unpublished data) the ^{14}C-bicarbonate must be removed in order to determine the protein bound radioactivity. Plasma and whole blood radioactivity on Days 1 and 2 were determined after acidification with 0.3 ml of concentrated hydrochloric acid in a hood prior to oxidation.

Results. The loss of radioactivity from the blood of four beagle dogs is shown in Fig. 1. The average apparent half survival times ($T_{½}$) by the ^{51}Cr and ^{14}C-cyanate methods were 19.2 $\pm$ 2.5 and 27.5 $\pm$ 1.1, respectively. In contrast with the ^{51}Cr data, the ^{14}C data yields a straight line ($r = -0.98$) when plotted on linear coordinates, further emphasizing the irreversible nature of the carbamylation reaction. The percentages of ^{51}Cr and ^{14}C radioactivity in the plasma at 24 hr after the reinfusion into the animals were 1.5 and 4.3%, respectively.

The sample taken 24 hr after the reinfusion was found to be more reliable as a 100% value than the 30 min aliquot, since a sudden loss of 15–20% of the radioactivity was frequently seen on the first day after the reinfusion. This sudden disappearance of radioactivity probably reflects cellular damage during the labeling process and was not observed when the ^{14}C-cyanate was administered intravenously. In fact, the labeling of erythrocytes *in vivo* by the intravenous administration of cyanate probably gives a better reflection of erythrocyte survival since it requires no *ex vivo* handling of the cells. This hypothesis is also supported by the fact that the measurement of erythrocyte survival by the *in vivo* ^{14}C-cyanate labeling method in two beagle dogs gave a loss of radioactivity which was linear with respect to time from 30 min after the infusion, and had an increased $T_{½}$ of 31.5 days. The intravenous infusion of 100 μCi of ^{14}C-cyanate into the dogs resulted in approximately 2.5×10^4 dpm/ml of total blood at 30 min. Of this value, 11% was due to the carbamylation of serum proteins and the remainder of the radioactivity was accounted for as a specific reaction with the amino-terminal valine of hemoglobin (3). The red cell membrane accounted for only 0.14% of the ^{14}C radioactivity. The contribution of the ^{14}C labeled serum proteins was subtracted at each time point from the whole blood radioactivity.

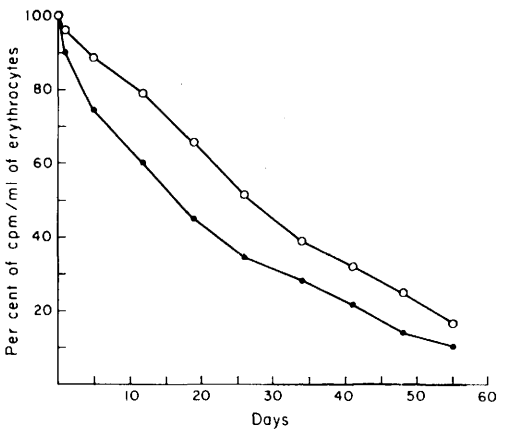

FIG. 1. Average erythrocyte survival curve for four beagle dogs as measured by ^{51}Cr (●) and ^{14}C-cyanate (○) *in vitro*.

TABLE I. Comparison of RBC Half-Lives (Days) as Measured by ^{51}Cr and ^{14}C-Cyanate.

Monkeys	^{51}Cr	^{14}C-Cyanate
A	14.8	18.0
B	16.0	19.6
C	16.0	18.8
D	15.8	20.8
Av	15.6	19.3

The apparent ^{51}Cr and ^{14}C-cyanate $T_{½}$ values with erythrocytes labeled *in vitro* for a series of four rhesus monkeys are recorded in Table I. The apparent ^{51}Cr $T_{½}$ are in accord with the value of 14.7–17 days reported by Mulder, Brown and Corwin (8). As in the dog, the apparent ^{14}C-cyanate $T_{½}$ is longer than the ^{51}Cr $T_{½}$, and probably is a better estimate of erythrocyte survival.

The *in vitro* and *in vivo* ^{14}C-cyanate method was also compared with the ^{51}Cr method in the basenji dog with hereditary pyruvate kinase deficiency of the erythrocyte. The fol-

lowing $T_{½}$ values were observed: (a) *in vitro* ^{51}Cr $T_{½}$ = 1.5 days; (b) *in vitro* ^{14}C-cyanate $T_{½}$ = 3.5 days; (c) *in vivo* ^{14}C-cyanate $T_{½}$ = 4.7 days. The apparent $T_{½}$ values observed with the ^{51} Cr method approximates the value of 2.5–3.0 days reported by Searcy, Miller and Tasker (5) for similarly afflicted dogs with this method. The *in vivo* labeling of erythrocytes with ^{14}C-cyanate probably gives a better estimate of erythrocyte survival in this severe hemolytic anemia. In contrast to the small amount of acid precipitable ^{14}C radioactivity found in the plasma of normal dogs, monkeys, mice or rats (unpublished data) after injection of ^{14}C-cyanate, the plasma of the anemic basenji incorporated large amounts (72%) of the total blood radioactivity at 30 min. Most of this radioactivity was found to be associated with the albumin fraction when the serum was subjected to starch block electrophoresis. It is not known why the albumin from the anemic animal should react more than that of normal animals.

Discussion. The irreversible nature of the reaction of cyanate with the amino-terminal valine of hemoglobin to form a carbamyl group makes cyanate useful as a method for introducing a radiolabel into a mixed age population of erythrocytes. The ^{14}C-carbamyl group is not reutilized and in the dog is excreted in the urine as carbamylated dipeptides (unpublished data).

The relative specificity of cyanate for the amino-terminal valine of hemoglobin when administered directly to experimental animals is quite striking and makes it possible to label the erythrocytes *in vivo* without having to manipulate the cells *in vitro*. The infused cyanate does react with other proteins in the animal, but in most cases it reacts predominantly with the hemoglobin within the first few minutes. Approximately 75% of the infused cyanate is metabolized within the first 6 hr to form carbon dioxide and ammonia; therefore, attempts should be made to place the animal in a hood or well-ventilated area during this period after infusion. The radioactive carbamyl groups associated with other proteins in the animal are eventually eliminated in the urine. In the species studied in the present communication the erythrocytes had only one hemoglobin type. Before the ^{14}C-cyanate method can be extended to situations with more than one hemoglobin type, such as man, it would first have to be shown that the different hemoglobins involved react with cyanate at about the same rate and extent.

Summary. The recent finding that cyanate reacts specifically with the amino-terminal valine of the hemoglobin molecule in an irreversible carbamylation reaction has led us to develop a ^{14}C-cyanate method for determining erythrocyte survival. The procedure is advantageous in that erythrocytes can be labeled either *in vitro* or *in vivo*.

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