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STUDIES OF HEME METABOLISM USING THE ENDOGENOUS PRODUCTION OF CARBON-14 LABELED CARBON MONOXIDE

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Contents

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ABSTRACT

It has been amply demonstrated that carbon monoxide is produced in man and other mammals solely from the catabolism of heme, and that 1 mole of CO is produced and rapidly excreted intact in the breath for every mole of heme degraded. The origin of this CO appears to be the alpha-methene bridge carbon atom of heme: this carbon atom can be labeled with carbon-14 by use of the specific biologic precursor glycine-2-^{14}C. Under such circumstances, the production of ^{14}CO in the breath reflects the destruction of labeled hemes within the body.

A method is presented for the detection and quantitation of ^{14}CO production in rats and mice, following glycine-2-^{14}C injection. The method includes simultaneous and continuous measurement of $^{14}CO_2$ production through use of an ionization chamber. The $^{14}CO_2$ is subsequently removed from the air stream by means of soda lime, the ^{14}CO is oxidized to $^{14}CO_2$ by means of a Hopcalite catalyst, and the $^{14}CO_2$ thus generated is absorbed in alkali and counted by liquid scintillation. This method is shown to give quantitative recovery of ^{14}CO without significant contamination from $^{14}CO_2$ or other volatile compounds from the breath, urine, or feces.

The production of labeled CO more than 3 days after injection of labeled glycine is analyzed by using a mathematical model. This model is shown to be generally applicable in the experimental animals used, in the presence or absence of erythropoiesis, and also in states in

which erythropoiesis is deranged. Labeled CO production rates, when fitted to the functions herein described, by the use of a digital computer, yield accurate estimates of the rate of random hemolysis, the mean potential lifespan and the spread of lifespans about this mean, and the fractional incorporation of the injected glycine into RBC hemoglobin heme. In addition, the production of ^{14}CO due to the processes responsible for the "early labeled peak" of CO production, and that due to the persistence of label in the glycine pool are also estimated. From these results, the mean overall erythrocyte lifespan, and the percent of red blood cells dying of senescent processes can be derived. This technique is shown to be independent of any steady state restrictions, and is able to reproduce the results obtained by the more conventional red cell labeling techniques without the use of repeated venesection. Definition of the parameters of RBC destruction appears to be more accurate than possible with previously available techniques, and variations of as little as 5 percent in the mean potential lifespan can be accurately determined and interpreted.

This method is applied to RBC survival in mice and rats following graded erythropoietic stimulation from hypoxia, bleeding, erythropoietin injection, or phenylhydrazine-induced anemia. Results indicated that, despite increases in erythropoietic rate up to 10 times normal by such stimuli, there was no evidence for increased "ineffective erythropoiesis" or the production of a double population of RBC by such stimuli, with the exception of rats studied shortly after the last of three doses of phenylhydrazine, suggesting a toxic effect of the drug during this period of time. A modest but significant shortening of the mean potential lifespan was seen, which increased linearly with increasing hemoglobin synthesis rate. This shortening never exceeded 35 percent, and no shortening of any significant degree was seen unless estimated hemoglobin synthesis rates were more that 2-3 times normal. The minimal alterations in RBC survival noted in these experiments despite marked erythropoietic stimulation lead one to conclude that these cells are remarkably normal.

I. ENDOGENOUS PRODUCTION OF CARBON MONOXIDE AND HEME CATABOLISM

A. General and Historical Remarks

Although there were numerous reports in the world literature concerning the presence of a combustible gas in the blood and breath of man and other mammals in the late 19th century (1, 2), it was not until the 1940's that it was conclusively shown to be carbon monoxide. In that decade, Roughton and Root (3) showed that there was a small, but measurable amount of CO in normal human blood. Later, Sjöstrand in Sweden confirmed these findings, and was able to show that the CO concentration was higher in expired air than in inspired air, establishing that the CO was actually produced within the body. He estimated normal CO production to be approximately 0.5 to 1.0 ml per hour in the normal adult female (4), values which are in close agreement with those accepted at the present time.

At the same time, Sjöstrand noted that endogenous production of CO was higher in patients with hemolytic anemias, extensive trauma, or following certain surgical procedures or mismatched transfusions (4, 5). These findings suggested that CO production was intimately associated with the decomposition of hemoglobin. In 1952, Sjöstrand showed that solutions of hemoglobin and myoglobin liberated CO on standing, and that maximal production of CO from such solutions corresponded to the CO-binding capacity of the initial solution (6). Injections of hemolyzed blood and hemoglobin solutions into humans, dogs, and rabbits also caused increased endogenous production of CO *in vivo*, and the increased CO production was noted to be approximately 1 mole of CO for every mole of heme injected (7, 8).

It had long been known that the bile pigments were end-products of hemoglobin catabolism within the body, arising specifically from heme, the prosthetic group of hemoglobin. The main difference between the heme and bilirubin molecules is the loss of the alpha-methene bridge carbon atom of the former, resulting in the formation

of a linear tetrapyrrole (bilirubin) from a cyclic tetrapyrrole (heme). Libowitzky and Fischer (10) were unable to recover this missing one-carbon fragment in vitro as either formic acid or formaldehyde, however. On the basis of his work, Sjöstrand suggested that the missing carbon atom was incompletely oxidized to CO, and excreted intact in the breath. This finding was later confirmed and amplified by Ludwig, Blakemore, and Drabkin in 1957, using labeled heme (9).

Following the demonstration that CO production in nonsmokers was directly related to hemoglobin breakdown within the body, numerous studies appeared in the literature in the 1950's concerning the relationship between the level of CO in the blood (as carboxy-hemoglobin) and hemoglobin catabolism, and this measurement became clinically useful in the diagnosis of hemolytic states. In 1957, Engstedt produced a fine monograph in which a high correlation was found between CO-HGB levels and reticulocyte count, ^{51}Cr RBC survival, and fecal stercobilin production (11).

In the 1960's, continuing the work originally started at the University of Pennsylvania by Ludwig, Blakemore, and Drabkin in the 1950's Coburn, Forster, and co-workers published a series of papers concerned with the accurate determination of CO-HGB levels, and developed a rebreathing method for determination of CO production rates in animals and man (12-16). With this method they showed that normal man produces approximately 0.4 ml of CO per hour, and that this amount was exceeded in various hemolytic states. By injecting solutions of hemolyzed blood, damaged RBC, heme, hemoglobin, and other tetrapyrroles, they were able to account for the production of 1 mole of CO per mole of injected heme, thus confirming in every respect the earlier studies of Sjöstrand and his co-workers. However, they noted that the normal endogenous CO production rate was higher than that calculated from the known amounts and turnover rates for circulating RBC hemoglobin, being about 20-30 percent higher than expected (12, 16). Earlier studies of bile pigment metabolism (17, 18) had amply shown that there was a source of bile pigments distinct from the breakdown of heme from circulating RBC hemoglobin, and that this source accounted for

approximately 10-25 percent of total bile pigment production. These sources were postulated to be:

(a) Myoglobin heme catabolism

(b) Catabolism of other heme-containing enzymes (cytochromes, catalase, etc.)

(c) Excess production of heme in marrow and other sites

(d) Production of bilirubin through anabolic pathways

(e) Early death of RBC within the marrow or shortly after their release (ineffective erythropoiesis)

(f) Scarf of hemoglobin around the extruded nucleus of the normoblast (Bessis, 19).

Using tracer studies, Coburn and co-workers demonstrated that labeled CO was produced within the first few days following injection of glycine-2-^{14}C, which specifically labels the alpha-methene bridge carbon atom of heme. The amount of labeled CO recovered in this time period was of the correct order of magnitude to account for this excess CO (20). Similar results were reported by Landaw and Winchell in studies of heme catabolism in rodents (21). In addition, White et al. demonstrated in vitro production of CO from liver slices with the simultaneous production of bilirubin (22), confirming earlier studies by Ibrahim, Schwartz, and Watson (23) and Robinson, Owen, Flock, and Schmid (24) that nonhemoglobin hemes in the liver are important sources of bile pigment. Schwartz, Ibrahim, and Watson (25), Berlin (personal communication), and Landaw (26), using hypertransfused study animals in which erythropoiesis was nearly 100 percent suppressed, estimated that about 40 percent of the early-appearing bile pigment (and CO) was due to catabolism of nonhemoglobin hemes. Recently, direct evidence of increased CO production in states associated with increased ineffective erythropoiesis has been obtained (26, 27).

At this same time there grew rather slowly an appreciation of the importance of this endogenously produced CO in clinical and experimental conditions. It was noted that in patients undergoing anesthesia using rebreathing circuits that CO levels within the anesthesia apparatus often exceeded 50-100 parts per million (28), long considered toxic

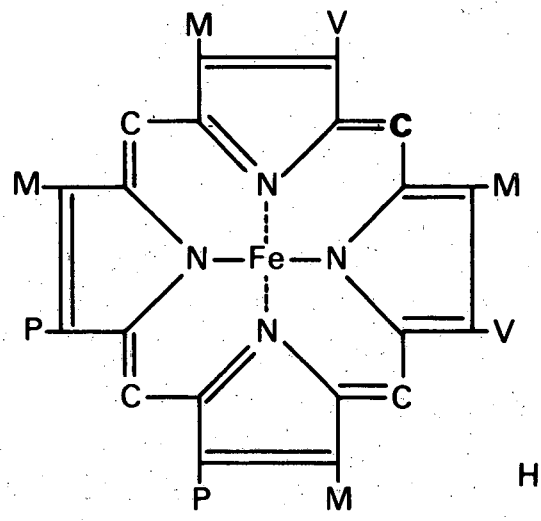
levels for industrial workers. The authors suggested that these closed rebreathing systems be opened and flushed periodically during the operative procedure in order to remove this excess CO. Increased CO-HGB levels were also seen in newborn infants (29, 30) due to normal and abnormal hemolysis. The endogenous production of CO in these newborns led to markedly increased CO-HGB levels (up to 12%), leading to critically lowered O_2 availability.

It had long been known in undersea operations that CO arising from internal combustion engines, tobacco smoke, and other sources were an important source of atmospheric pollution in submarines. For this reason, catalytic burners were provided to oxidize CO to harmless CO_2, which contributed greatly to the increased effectiveness of the submarines in remaining submerged for long periods of time without producing severe symptoms suggestive of CO toxicity in the crew. However, in closed systems containing men or animals in which there was no other known source of CO, the puzzling production of CO was soon noted, leading often to toxic or fatal CO levels in some animal experiments in which this contaminant was not specifically removed from the atmosphere. This rather unexpected situation produced a great awareness of the crew member as an important source of pollution of closed systems, and greater attention was turned towards the endogenous production of CO in closed systems being evaluated for space flights and undersea exploration (31). It is conceivable that with the use of animal-plant cycles that have been proposed for long space flights for the production of O_2 and removal of waste products, that an additional source of CO production may be found in the decomposition of the green respiratory pigment of plants, chlorophyll, which contains a cyclic tetrapyrrole structure similar to heme, since mature leaves have been shown to produce large quantities of CO <u>in vivo</u>, presumably from degradation of this pigment (32). In this context it might be of interest to mention the possibility that a CO cycle exists in nature, since it has been demonstrated that many animal and plant species produce this compound (32-36) while it can be utilized for metabolic purposes in certain bacteria (37) and plants (38), and may even be oxidized to CO_2

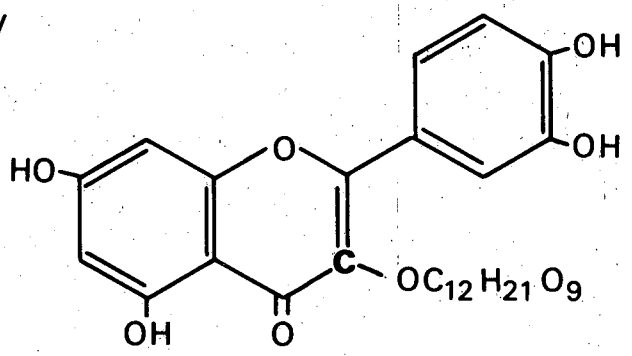
at slow rates in animals (39) and man (40).

B. Biochemistry of Heme Catabolism

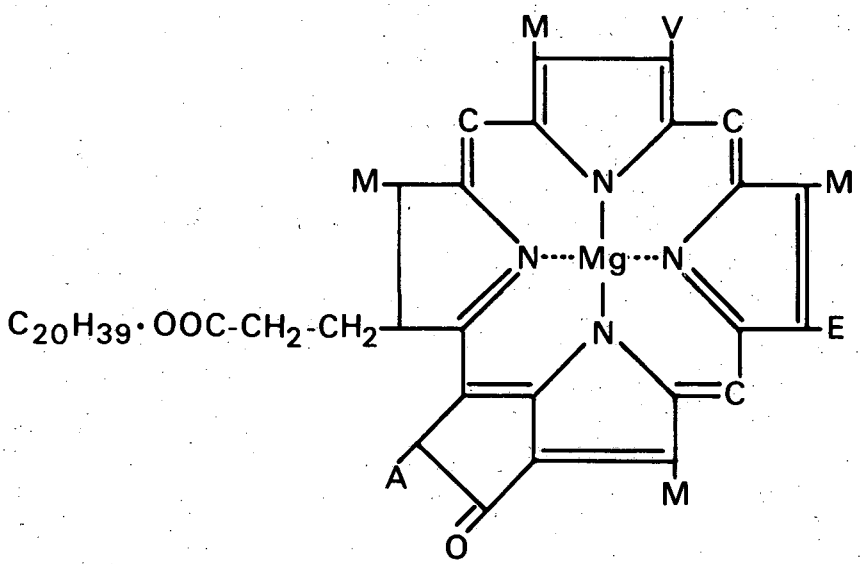
Heme, a cyclic tetrapyrrole derived from the protoporphyrin IX structure, is shown in Fig. 1. It is degraded within the body by an incompletely understood mechanism, the first step of which requires the opening of the cyclic tetrapyrrole at the alpha-methene bridge carbon atom. Although specific enzymes have been postulated for this step, or series of steps (41-43), it is possible that nonspecific oxygenases can also be effective in vivo, while radiation and many chemicals are also capable of breaking open the cyclic structure in vitro (7, 9,43). The one-carbon fragment is then presumably split off from the molecule, with the production of bilirubin and CO. Pullman and Perrault (44) have shown, using molecular orbital theory, that the alpha-methene carbon atom has the greatest π electron charge density of the four bridge carbon atoms, so that it is the one most easily oxidized. It is not known why this particular one-carbon fragment is handled differently from all other one-carbon fragments in the body, but thus far the endogenous production of CO in mammals seems to be exclusively from the catabolism of heme. CO is also produced by plants that contain chlorophyll, and since this compound is also a tetrapyrrole, the mechanism for CO production may be similar to its production from heme. However, CO is also produced during the degradation of rutin, which does not have the tetrapyrrole structure (34) (Fig. 1). The pneumatosaccus of the Portuguese Man-of-War, which produces large quantities of CO, apparently produces this gas utilizing serine as a precursor. The folic acid content of this gland is 10 times that of mammalian tissues, so that one-carbon fragment transport and metabolism is strongly implicated in the production of CO by this organism (36). In the Brown alga (Egregia menzies) CO is also produced, in a process that may be O_2 dependent. It has been suggested that a polyphenolic compound such as gallic acid or pyrogallol may be the immediate precursor for this CO (33). The only common denominator for the compounds mentioned, with the exception of serine, appears to be a highly



HEME (ANIMALS)



RUTIN (BACTERIA)



CHLOROPHYLL a (PLANTS)

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Fig. 1. This figure depicts three compounds which have been shown (or inferred) to be precursors for endogenous production of CO in animals, plants, and bacteria. The carbon atoms known to be oxidized to CO are shown in boldface type. Note the similarity between the structure of heme and chlorophyll. (M: methyl, V: vinyl, P: propionic, Ac: acetyl, E: ethyl.)

unsaturated polycyclic compound. It is entirely possible that vitamin B_{12}, which also has a cyclic tetrapyrrole structure, may be biologically degraded to CO, although evidence for this has not yet been presented. Thus, in spite of numerous studies, one must conclude that the mechanism(s) for the production of CO from biologic compounds is almost entirely unknown, and we must satisfy ourselves that, in the mammal, endogenous production of CO seems to arise solely from the catabolism of heme. Previous suggestions that CO arises in man from degradation of sugars or oxalic acid (45) have thus far not been confirmed. Recent work by Callahan, Landaw, and Schmid (46) has shown, within experimental error, a 1:1 molar production ratio of bilirubin and CO in normal rats, and the production of labeled CO following the injection of glycine-2-^{14}C seems to be entirely accounted for by the degradation of labeled heme. The "excess CO" noted by Coburn and co-workers (12) is of the same magnitude that would be expected from the degradation of nonhemoglobin hemes, so that an additional source for CO does not seem likely. However, in recent work, Nishibayashi, Omura, Sato, and Estabrook (47) have suggested that CO may be produced during lipid peroxidation by liver microsomes in vitro, although the source of this CO has not yet been elucidated.

More than 90 percent of the heme contained within the mammalian body is contained within the hemoglobin of mature circulating red blood cells, while the amount contained in myoglobin has been estimated as about 5 percent of the amount in hemoglobin. The total mass of heme associated with the other hemoproteins which are present in all cells constitutes an important but extremely small fraction of total body heme. However, because of their very high turnover rates, some of these hemoproteins, especially those of the liver, appear to contribute significantly to the endogenous production of CO in normal mammals, probably accounting for about one-half of the "excess CO", or about 10 percent of total CO production.

A normal 70-kg man has a total circulating blood volume of approximately 5 liters, and a circulating hemoglobin mass of approximately 750 grams. Assuming a molecular weight for hemoglobin of

68,000, this equals approximately 44 millimoles of circulating hemoglobin heme. Since the average RBC lifespan is approximately 120 days in man, 0.83 percent of this amount of heme is degraded per day, or 350 micromoles. At STP, this equals approximately 0.33 ml of CO per hour. As mentioned previously, this is about 20 percent lower than the amount of CO produced in normal man by the method of Coburn and Forster (0.42 ± 0.07 ml per hour) (12). It is of interest that the "excess CO" in normal dogs is about 40 percent more than predicted (48), while the amount for the mouse and rat is predicted to be about 25 percent (26). It appears that this variability in "excess CO" is a manifestation of the turnover rates of nonhemoglobin heme compounds within the liver and probably differs for each mammalian species. Under conditions in which there may be excess breakdown of heme compounds in the liver (or muscle), one might then expect marked increases in this "excess CO." Such increases have been found in animals and man with porphyria (26, 27), and following the administration of phenobarbital (46, 49), which stimulates ALA-synthetase activity in the liver, the rate-limiting step in porphyrin synthesis. Coburn found that CO production was more than doubled following phenobarbital treatment of normal volunteers (49), from a mean of approximately 17 micromoles per hour to 38 micromoles per hour. Following cessation of treatment the "excess CO" decreased with a half-time of approximately 2 days, which approximates the turnover rate of "rapidly turning over tissue iron" (Winchell, H. S., personal communication) in studies of normal man. In hemolytic states, CO production may be more than 8 times normal (16), and elevations of this magnitude may also be found in patients with a high degree of "ineffective erythropoiesis" (27).

C. Physiologic and "Public" Health Aspects of Endogenous Production of Carbon Monoxide

As mentioned previously, the endogenous production of CO is of great importance in closed rebreathing systems, in which men or animals may be present for extended periods of time. From the normal

rate of endogenous CO production of approximately 0.4 ml per hour (12), one may calculate that, in a closed system in which CO is produced solely from heme catabolism, the concentration of CO will increase at a rate of 18 ppm per day if each man is given an average of 20 cubic feet of air space. At this time, toxic levels of CO (50 ppm or greater) will be achieved in less than 3 days. It is thus obvious that any or all of the following conditions will drastically reduce the time required for the production of toxic or lethal CO concentrations:

(a) Increased CO production due to disease processes

(b) Decreased air space per man, or

(c) Additional CO sources.

Studies performed in a closed system space cabin simulator, in which 4 men remained for up to 14 days, showed that CO concentration increased at a steady rate, and reached a maximum of 19 ppm at the end of the study (50). The rate of increase of CO concentration calculated from the data indicates the production of 0.37 ml of CO per man per hour, values which agree remarkably well with those of Coburn et al.

Preliminary data from a 45-day undersea saturation dive study (Sealab II) conducted at a pressure of 7 atmospheres, in which 10 men were confined to a space of 4500 cubic feet, showed that atmospheric CO concentration rose at a rate of 1.4 liters of pure CO per day (51). This is equivalent to the production of 5.8 cc of CO per man per hour, which is greatly in excess of that predicted from endogenous CO production alone. However, there were other sources for CO in this study (cooking was allowed, for example). A maximum CO concentration of 30 ppm was reached in this experiment, but the initial rate of increase of CO concentration was approximately 1.6 ppm per day.

CO concentrations as high as 340 ppm were found in a closed rebreathing anesthesia apparatus (28) in nonsmoking individuals being operated upon for various disorders. It is obvious that under these conditions CO build-up may be even more dangerous since the patients are not awake and cannot express symptoms due to the accumulation of CO.

It had been shown by Metz and Sjöstrand in 1954 that CO concentrations actually decreased with time in closed systems containing experimental animals in which CO-HBG levels were higher than 4-5 percent. They suggested that this may have been due to oxidation of CO to CO_2 within the animal (52). Recently, Luomanmäki found that dogs breathing in a closed system oxidized CO to CO_2 at a rate of approximately 0.3 percent per hour (40). In two normal human volunteers the rate of CO oxidation was found to be 0.11 and 0.16 percent per hour, and the absolute amount of CO oxidized was calculated to be 0.02 ml per hour. Luomanmäki calculated that at a CO-HBG level of 21.5% the endogenous production of CO would be balanced by an equal amount of oxidation of CO, thus producing a steady state. A decrease in CO concentration was actually noted in Sealab II after about 28 days, and it is entirely possible that oxidation of CO to CO_2 within the bodies of the divers may have been an important factor in this decrease.

Recently studies by Coburn et al. (48) and White et al. (22) have shown that high concentrations of exogenous CO inhibit the production of CO from heme both <u>in vitro</u> and <u>in vivo</u>. In dogs with CO-HBG levels of 11-13 percent, recovery of injected hemin-^{14}C as labeled CO was only 35-42 percent, whereas recoveries of approximately 97 percent were achieved in dogs with normal CO-HBG concentrations (48). Although studies to determine the site of action and possible long-term effects of high CO-HBG levels on hemin catabolism have not yet been performed, it is possible that high concentrations of CO in the atmosphere may exert a significant physiologic effect through such pathways.

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II. ENDOGENOUS PRODUCTION OF ^{14}CO: AN *IN VIVO* TECHNIQUE FOR THE STUDY OF HEME CATABOLISM

A. Introduction

In the late 1940's, Sjöstrand demonstrated the presence of carbon monoxide in the expired air of the normal, nonsmoking man (1). By *in vivo* and *in vitro* techniques he was able to show that this carbon monoxide was produced during the catabolism of heme, the important prosthetic group of hemoglobin, in the ratio of one mole of carbon monoxide per mole of heme catabolized. Later, Ludwig, Blakemore, and Drabkin (2, 3) showed that the α-methene bridge carbon of heme was the direct source of this carbon monoxide. These and other findings concerning CO production and heme catabolism were reviewed in Chapter I.

It has been established that the metabolic precursor of the four methene bridge carbon atoms of heme is the methylene (number 2 carbon) carbon of glycine (4), and several investigators have been able to recover carbon-14 labeled carbon monoxide in the expired air of mammals, including man, following injection of glycine-2-^{14}C (2, 3, 5, 6). Since it has been adequately demonstrated that endogenously produced carbon monoxide can be detected and/or recovered with efficiencies approaching 100% in man (7), other mammals (8), and *in vitro* systems (9), it was expected that an isotopic technique using the above findings would allow quantitative, continuous, *in vivo* study of heme catabolism by measuring the rate of production of ^{14}CO following the injection of glycine-2-^{14}C.

B. Methods and Instrumentation

Standardization of Radioactive Gases

Standard tanks of ^{14}CO and $^{14}CO_2$ were obtained by adding the labeled gases (New England Nuclear, Boston, Mass.) to a measured volume of air containing carrier CO and CO_2, respectively, in pressurized cylinders. Exact determination of the concentration of radioactive gas in the cylinders (μCi/liter) was simultaneously performed

by two separate methods.

Method I: Ionization-Chamber Standardization. The ^{14}CO and $^{14}CO_2$ standard gases were passed through a 22-liter ionization chamber operating at atmospheric pressure, with a collecting potential of 300 V. The signal from this chamber was then passed to a vibrating reed electrometer (Model 30, Applied Physics Corporation, Pasadena, Calif.) fitted with a 10^{12}-ohm precision resistor (Victoreen Company, Cleveland, Ohio). The resulting voltage was recorded on a recording potentiometer.

Tolbert (10) has shown that ionization chambers of this size have counting efficiencies for the beta particle of carbon-14 approaching 100%, with a very high stability over a period of months. He calculated a theoretical calibration constant of 1.24×10^{-4} μCi/mV for a 10^{12}-ohm resistor. A signal of 1 mV from a chamber of this size means that the total activity within the chamber is 1.24×10^{-4} μCi, with a concentration of activity of 5.64×10^{-5} μCi/liter. The results obtained for the two standard gases are shown in Table 1a.

The method just outlined is subject to various errors, some of which are not easily ascertained. These include: error in the calibration of the precision resistor (± 2%), error of reading the potentiometer signal, changes in ambient temperature and atmospheric pressure, plus any deviation from ideality in the counting efficiency. In addition, the calculation of the calibration constant requires accurate values for the average energy of the carbon-14 beta particle and the average ionization potential for air. Thus, the values calculated in Table 1a are probably accurate only to within 5 to 10%.

Method II: $^{14}CO_2$ Absorption in Alkali and Liquid Scintillation Counting.

The $^{14}CO_2$ standard gas was studied by absorption of known volumes of the gas in an alkali solution, and was subsequently counted by liquid scintillation techniques. The method of Jeffay and Alvarez was used without modification (11). A measured amount of the $^{14}CO_2$ standard gas was absorbed in 13 ml of a 1:2 (v/v) solution of ethanolamine in ethylene glycol monomethyl ether. Three milliliter aliquots of this absorber solution were added to 15 ml of a scintillator solution made

Table 1. Calibration of the ^{14}CO and $^{14}CO_2$ standard gases.

Table 1a

Ionization chamber calibration of the ^{14}CO and $^{14}CO_2$ standard gases

Standard gas	Maximum net millivolts recorded	Calculated activity concentration (μCi/liter)
(A) $^{14}CO_2$	9.80	5.53×10^{-4} (A)
(B) ^{14}CO	137.00	7.73×10^{-3} (B)

Table 1b

Alkali absorption and liquid scintillation calibration of the $^{14}CO_2$ standard gas (A)

Liters of standard $^{14}CO_2$ gas (A) passed through absorber solution	Net DPM absorbed	Calculated activity concentration (μCi/liter)
15.00	17,620	5.29×10^{-4}
16.00	19,600	5.52×10^{-4}
17.00	20,310	5.38×10^{-4}
Total: 48.00	57,530	5.40×10^{-4} (A)

of a 1:2 (v/v) solution of ethylene glycol monomethyl ether in toluene, containing 5.50 g/liter of 2, 5-diphenyloxazole (PPO, Scintillation Grade, Packard Instrument Company, Downers Grove, Illinois). The resulting homogenous colorless solution was then counted at 0° C in a Nuclear Chicago Model 725 Liquid Scintillation Counter. Internal standardization was achieved with a standard toluene-^{14}C solution (3.83×10^5 dpm/ml, New England Nuclear, Boston, Mass.). Typical counting efficiencies (cpm/dpm) were in the range of 0.497 to 0.510. Background ranged from 34 to 43 cpm. Table 1b shows the results obtained using this method. Standard ^{14}CO gas was found to be completely insoluble in the ethanolamine solution and could not be standardized by this technique.

The sources of error are more precisely known in this method than in the ionization chamber method. They include: estimated volumetric errors (0.5%), error in internal standard (2.4% for 99.5% confidence), and flowmeter error (0.5%). The values calculated in Table 1b are probably accurate to within 3%.

The value obtained for the $^{14}CO_2$ standard using the liquid-scintillation method agrees with the value determined by the ionization chamber method within 2.4%. The close agreement of the results from these two different techniques supports the accuracy of both methods and allows one to assume that the ^{14}CO standard activity is approximately that calculated from the ionization chamber method alone. These two standard gases were then used to test the breath collection system shown in Fig. 1.

In Vivo ^{14}CO Detection System (Fig. 1)

The metabolism cage is made of clear plastic, with a volume of approximately 2 liters. It can hold a 350- 450-g rat with ease and allows freedom of motion for the animal. Air enters through an intake port in the top. An additional opening (not shown) can be used to monitor the pressure within the cage with a water-filled manometer. Air exiting from the cage is dried by passage through anhydrous $CaSO_4$ (Drierite, Indicating, W. A. Hammond Drierite Company, Xenia, Ohio). The air then passes through a 22-liter ionization

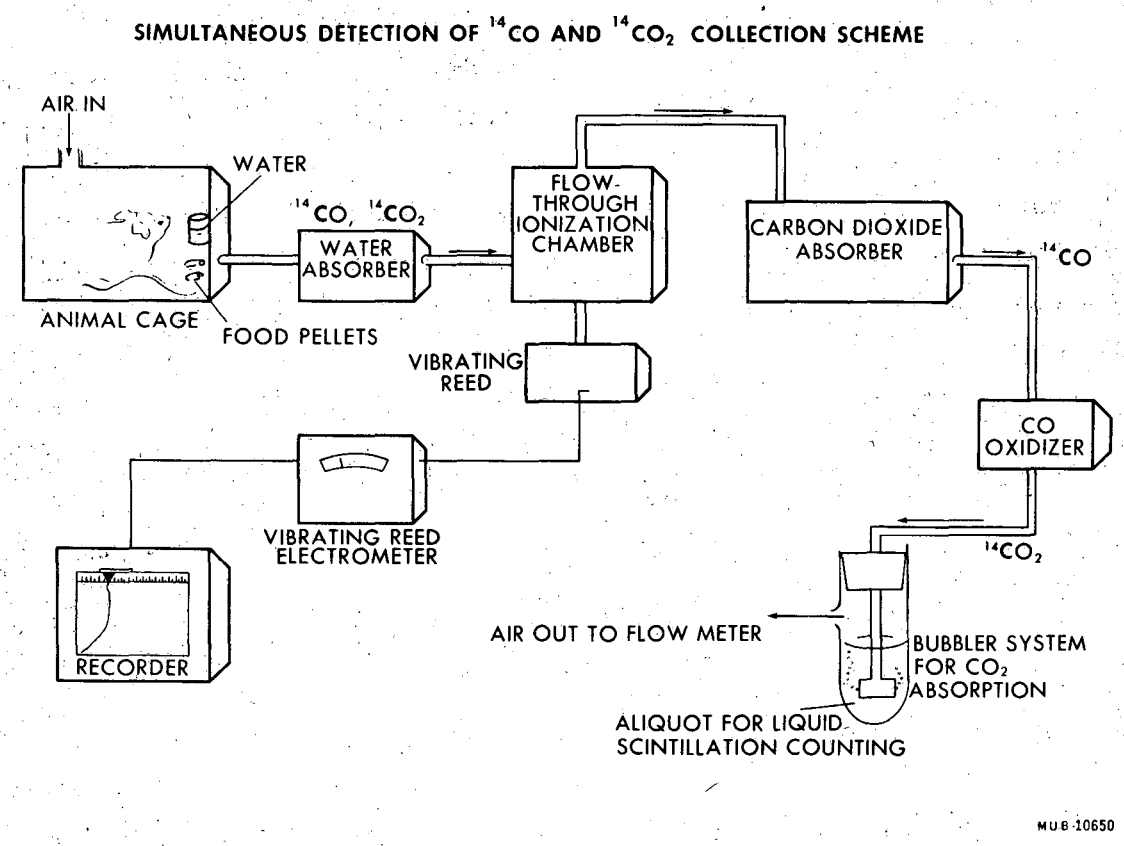

Fig. 1. In vivo breath collection system. Schematic representation of apparatus used to measure $^{14}CO_2$ and ^{14}CO production simultaneously and continuously in the intact rat. The radioactive gases present in the air stream are noted for three different portions of the apparatus. The separate components of the system are described in the text.

chamber system as previously described under Method I. Air is continuously evacuated from the ionization chamber by means of a constant-flow diaphragm pump (N. V. Godart, Holland). The air is then passed through a soda lime cannister containing approximately 250 g of sodium calcium hydrate and 100 g of Ascarite (Arthur H. Thomas Co., Philadelphia, Pennsylvania), which serves to remove the CO_2 and $^{14}CO_2$ from the air stream. Exhaustion of this cannister is detected by a color change in the Ascarite.

The air then passes through a cannister containing approximately 17 g of Hopcalite (Mine Safety Appliance Company, part No. 41566). Hopcalite is a trade name for a mixture of magnesium and copper oxides with other catalytic agents, and serves to oxidize CO to CO_2 at low ambient temperatures (12). Care must be taken to insure that the air passing through the Hopcalite is completely dry in order to obtain maximum oxidative efficiency. The air is then bubbled through a coarse sintered-glass gas dispersion tube into the ethanolamine-ethylene glycol monomethyl ether solution previously described. The air exiting from the absorption tube is passed through a wet-test meter (American Meter Company, Albany, New York) for accurate determination of flow rates.

Animals used for experimentation were male buffalo rats, weighing 340 to 370 g, corresponding to an age of approximately 3 to 4 months. Glycine-2-^{14}C was purchased in multiple-dose vials containing 50 μCi/ml in sterile saline (New England Nuclear, Boston, Mass.). Fifty microcuries were injected intravenously into each animal under light ether anesthesia. The animal was placed inside the metabolism cage immediately thereafter and breath collection started within 1 min of the time of injection. The animals were fully reactive, and radioactivity of the expired air was noted via the ionization chamber readings 3 to 5 min after placing the animal in the metabolism cage. The flow pump was set to deliver a flow of approximately 0.35 to 0.50 liters/min. At these flow rates the animals were apparently comfortable, and there was moderately vigorous bubbling in the gas absorber tube.

C. Technique Analysis

Absorption Efficiency of Ethanolamine Absorber for $^{14}CO_2$

For the first experiments, two or three gas absorber tubes were connected in series. However, it became apparent that no statistically significant activity over background was ever present in the second and third absorbers when there were at least 10 ml of absorber solution in the first tube. Therefore, all subsequent experiments were performed with a single collecting tube. From prior experimentation by Sjöstrand (8), and with an absorption capacity of the ethanolamine for CO_2 on the order of 2.67 millimoles per milliliters, it was calculated that 10 ml of solution would be sufficient for the collection of all the endogenously produced carbon monoxide in a small rodent for a length of time exceeding 1 week, if all the carbon monoxide is oxidized to carbon dioxide by the Hopcalite. Ten milliliters were sufficient to trap all of the radioactivity contained in more than 50 liters of the ^{14}CO and $^{14}CO_2$ standard gases.

Evaporation of the absorber solution was noted to be approximately 0.5 ml/hr at a flow rate of 0.4 liters/min. Since ethanolamine is a known quencher of radiation in a liquid scintillation system (11) while the ethylene glycol monomethyl ether is not, unequal evaporation of the components of the absorber solution causes a change in counting efficiency. When the change in counting efficiency caused by evaporation was measured, it amounted to less than 0.3% and was thus not corrected for.

For these experiments enough absorber solution was used so that the final volume after evaporation was between 12 and 13 ml. Unused absorber solution was then added to make the final volume 13 ml. Thus, a 3-ml aliquot represented 3/13 of the total absorbed activity. These aliquots were counted in the system mentioned previously for at least 200 min, and, when necessary, for longer periods of time to insure a maximum of 2% counting error. After subtraction of background counts, the net cpm were converted to dpm with the use of an internal ^{14}C-toluene standard, and this value was multiplied by the factor 13/3 to calculate the total absorbed activity. Production rates were stated

as cpm/hr or μCi/hr.

Oxidation Efficiency of Hopcalite for ^{14}CO

In order to test the oxidation efficiency of Hopcalite for ^{14}CO, varying volumes and concentrations of the standard ^{14}CO gas were passed through the entire system as shown in Fig. 1. Results are shown in Table 2. It is apparent that oxidation efficiency approaches 100% at the highest CO concentration (5,000 ppm). Unfortunately, in the procedure used to prepare the standards containing the smaller concentrations of CO, errors were introduced which led to lower actual concentrations of ^{14}CO than that calculated on the basis of dilution alone. Thus, the oxidation efficiencies shown in Table 2 for gases containing 25 and 5 ppm are minimum values. Experiments are now in progress to determine the oxidation efficiencies at these low concentrations with more accuracy. These experiments have been repeatedly performed over a 12-month period, with reproducible results, so that the oxidation efficiency seems to be rather consistent.

Carbon Dioxide Absorption Efficiency of Soda Lime Absorber

One of the important requirements of the system depicted in Fig. 1 is that the radioactive CO_2 must be quantitatively trapped in the soda lime, so that remaining activity in the air stream is due to ^{14}CO alone. To test this, varying volumes of standard $^{14}CO_2$ gas were passed through the system. If the soda lime absorber were 100% efficient, no counts above background should be noted in the ethanolamine absorber. A second standard $^{14}CO_2$ gas, with approximately 7.5×10^{-3} μCi/liter was made in order to determine this efficiency more accurately. Twenty liters of this standard $^{14}CO_2$ gas were passed through the system. If there were no absorption of the $^{14}CO_2$ by the soda lime, there would have been 38,400 cpm in the ethanolamine absorber. However, only 0.50 net cpm over background were detected (with a standard deviation of 0.36 cpm). Thus the soda lime absorber allows the passage of about 1 part in 75,000 of the $^{14}CO_2$. It will be seen in a later section that this very high efficiency is necessary for complete separation of ^{14}CO from $^{14}CO_2$ in the experimental animal.

Table 2. Hopcalite oxidation efficiency.

Experiment number	CO concentration (ppm)	Calculated oxidation efficiency (%)[b]
1	5000	97.8
2	5000	97.8
3	5000	96.4
4[a]	25	88.7
5[a]	5	83.9

[a]See text for discussion of errors.

[b]Oxidation efficiency expressed as % of previously calibrated ^{14}CO Standard Gas (B) activity concentration of 7.73×10^{-3} μCi/liter (Table 1a).

Animal Experimentation Results

Figure 2 shows results obtained with the system described during the first 5 days after injection of 50 μCi of glycine-2-^{14}C into a normal buffalo rat. Three important features are noted in this figure:

1. The excretion pattern of the total breath activity (ionization chamber readings) is distinctly different from the curve describing the activity absorbed in the ethanolamine. We may then conclude that each of the patterns reflects the excretion rate of a different radioactive substance, and that the activity present in the ethanolamine is not due to a constant or variable leak of a small fraction of the $^{14}CO_2$ past the soda lime absorber.

2. The total breath activity ($^{14}CO_2$ + "^{14}CO") is 10^3 to 10^4 times that of the activity absorbed in the ethanolamine ("^{14}CO"). Thus the very high absorption efficiency of the soda lime absorber previously noted is needed to achieve the desired separation. It may then be concluded that the ionization chamber readings are essentially a measure of $^{14}CO_2$ production rates, since the fraction of the total breath activity not caused by $^{14}CO_2$ is so small.

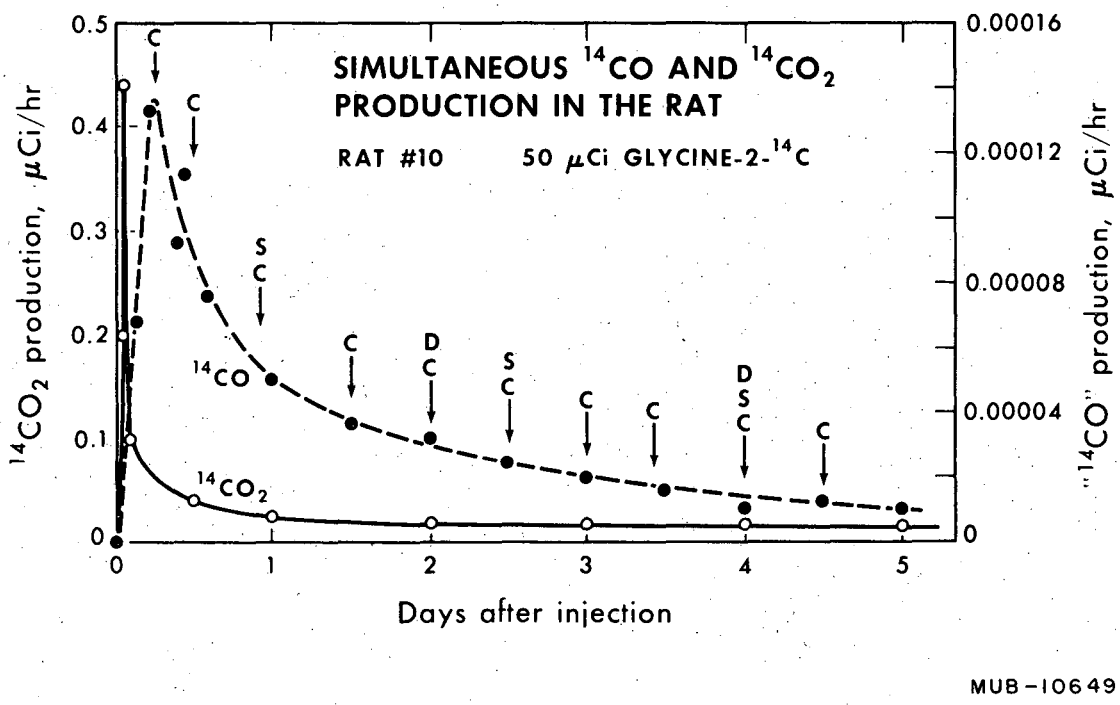

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Fig. 2. Simultaneous $^{14}CO_2$ and "^{14}CO" production in the rat. The graph shows the rate of appearance of $^{14}CO_2$ (open circles, left ordinate) and "^{14}CO" (closed circles, right ordinate) in the expired air of a normal rat following IV injection of 50 μCi of glycine-2-^{14}C, as obtained by the apparatus shown in Fig. 1. The factor of 3000 difference in the ordinate scales should be noted. Arrows refer to times when various components of the system were changed, as follows: C = Metabolism cage, S = Soda-lime cannister (CO_2 absorber), D = Drierite cannister (water absorber).

3. At the times noted on Fig. 2, the soda lime absorber, water absorber, and cage were replaced, without any significant change in the curve of activity absorbed in the ethanolamine ("^{14}CO"). One can thus conclude that no significant artifacts were due to contamination with volatile compounds from urine or feces (even though the urine and feces were found to be highly radioactive throughout this time period), or due to exhaustion of the soda lime and Drierite cannisters.

In order to estimate the magnitude of contamination of the "^{14}CO" with volatile compounds from urine, feces, and breath more precisely, three separate experiments were carried out. In the first, all the urine and feces excreted during the first 20 hr after injection of 50 μCi of glycine-2-^{14}C into a normal buffalo rat were placed inside a metabolism cage attached to the breath collection system of Fig. 1. Air was passed over this material for 5 hr at the usual flow rate (0.4 l/min), and the activity absorbed in the ethanolamine was noted. In the second experiment, 10 μCi of glycine-2-^{14}C was used in place of the urine and feces, while in the third experiment, 2 μCi of acetone-2-^{14}C was used. The results showed that evaporation of glycine (as might occur after glycinuria in the animal following the injection of a large mass of glycine) cannot account for more than 0.2% of the "^{14}CO" production, while the contribution from other volatile compounds in the urine and feces is 0.7% or less. In the case of acetone, only 0.008% of the activity placed inside the metabolism cage was absorbed in the ethanolamine. Thus it is unlikely that labeled acetone produced by a nonfasted animal can contribute significantly to the "^{14}CO".

Figure 3 shows the activity absorbed in the ethanolamine absorbers 30 to 90 days after injection of 50 μCi of glycine-2-^{14}C intravenously into a normal buffalo rat. The points approximate a Gaussian distribution between 60 and 75 days, with a mean of 66.5 days and a standard deviation of approximately 6.4 days. Since investigators in this laboratory, using independent techniques, have found a rat RBC life span on the order of 57 to 68 days (13, 14), it appears from this graph that the activity appearing in the ethanolamine is mirroring the senescence and destruction of the cohort of red blood cells produced at the time of

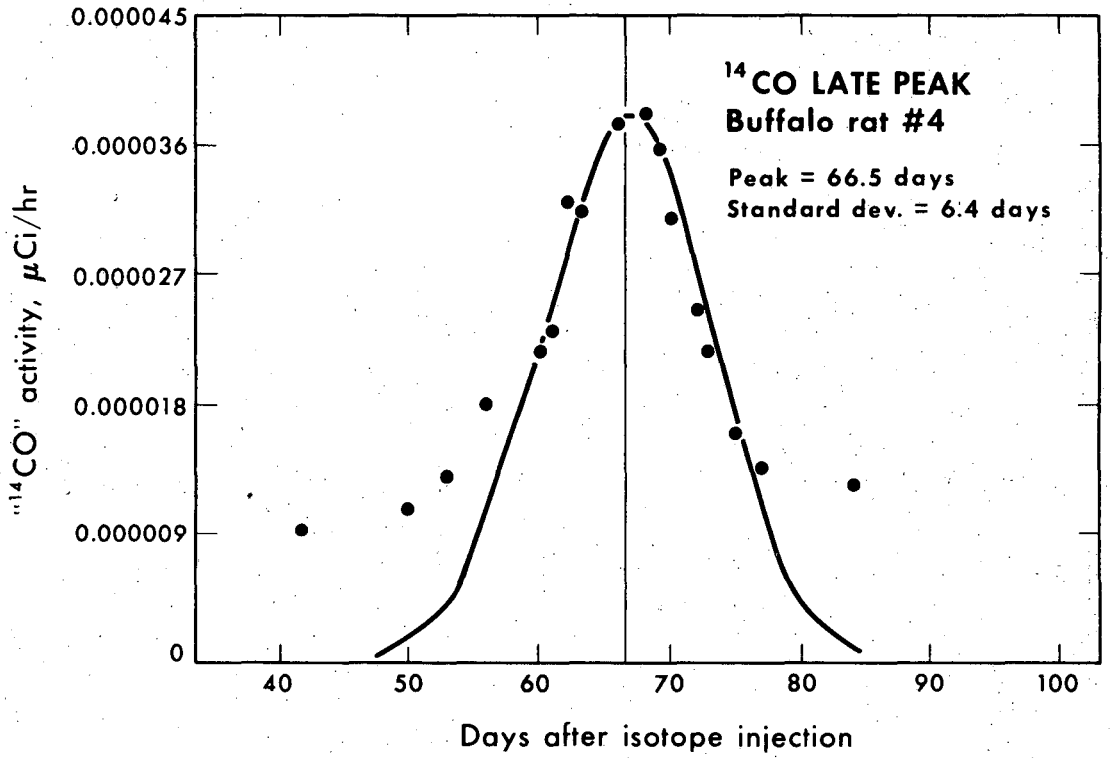

Fig. 3. "Late" production of "^{14}CO" in a normal rat. The rate of appearance of "^{14}CO" in the expired air 40 to 90 days after administration of 50 μCi of glycine-2-^{14}C to a normal rat is shown. Experimental points are indicated by closed circles. A "normal" or Gaussian curve is described by the heavy black line, having a mean of 66.5 days and a standard deviation of 6.4 days. These values represent an in vivo estimation of the distribution of RBC life spans about the mean value for a normal rat.

injection of the labeled glycine.

D. Discussion

The functioning of this system depends upon four important assumptions. They are: (1) Respiratory $^{14}CO_2$ can be completely absorbed in the soda lime cannister. (2) Breath activity remaining after $^{14}CO_2$ absorption is due solely to endogenously produced ^{14}CO. (3) ^{14}CO can be quantitatively oxidized to $^{14}CO_2$ by Hopcalite at ambient temperatures at the flow rates employed, and at the low concentrations present in mammalian breath. (4) The $^{14}CO_2$ produced from the oxidation of ^{14}CO can be quantitatively trapped in the ethanolamine solution and counted with known efficiencies.

These points will be discussed individually:

1. Figure 2 shows that the activity in the respiratory $^{14}CO_2$ is 1000 to 10,000 times as great as that simultaneously present in the component absorbed by the ethanolamine ("^{14}CO"). Eleven days after injection of the labeled glycine, the ratio of $^{14}CO_2$ activity to "^{14}CO" activity is approximately 800. The experiments with standard $^{14}CO_2$ gases showed that the soda lime absorber allows passage of about 1 part in 75,000 of the $^{14}CO_2$. Thus the efficiency of the soda lime cannisters is entirely sufficient for the desired separation.

2. Although it cannot be stated with absolute certainty from these experiments that the activity trapped in the ethanolamine is due solely to ^{14}CO produced by the catabolism of heme in the experimental animals, several points of evidence exist which strongly suggest that it is indeed due almost entirely to this source. These points are:

(a) Only three types of carbon-containing compounds are present in the expired air to any significant degree: CO_2, CO, and ketone bodies, all of which will be labeled with carbon-14 after injection of glycine-2-^{14}C. Experiments showed that only 1 part in 75,000 of the $^{14}CO_2$ excapes the soda lime absorber. Two of the ketone bodies, acetoacetic acid and beta hydroxy butyric acid, being acids, are likely to be absorbed by the soda lime with comparable efficiencies. It was also shown that only 1 part in 12,000 of acetone-2-^{14}C added to the system

is absorbed into the ethanolamine. Since the animals are not fasted during these experiments, it is unlikely that labeled ketone bodies exhaled by the animal can cause significant contamination of the "^{14}CO". Although recent studies of the atmosphere of closed systems have shown the presence of more than 50 carbon-containing compounds (15), separate experiments showed that contamination of the "^{14}CO" by volatile ^{14}C-containing compounds in the urine and feces cannot exceed 0.7%, while the contribution from glycinuria with evaporation of glycine-2-^{14}C cannot exceed 0.2%.

(b) Figure 3 shows that the production rate of the "^{14}CO" in one normal rat follows a Gaussian distribution about a mean value of 66.5 days. This result is to be expected from destruction of labeled heme in the circulating red blood cells of the rat, with subsequent production of ^{14}CO. Other experiments have shown that this peak production correlates well with the decrease in blood activity (16). This mean value also agrees well with estimates for the RBC life span of the rat, using other techniques. This phase of ^{14}CO production will be discussed in greater detail in Chapter 3.

(c) The amount of activity absorbed in ethanolamine during the first 5 days after injection of labeled glycine is equivalent to 15-30% of the activity incorporated into the α-methene bridge carbon of heme of circulating red blood cells in the rat (17). This "early appearing" material thus correlates well (in time of appearance as well as quantitatively) with the production of "early labeled" bilirubin (18, 19) and stercobilin (20) in the rat, dog, and man. Since bilirubin, stercobilin, and CO are all breakdown products of heme, the correspondence is significant, although not conclusive.

(d) The magnitude of this "early appearing" material ("^{14}CO") was found to be increased when erythropoiesis was stimulated in the rat after phenylhydrazine treatment or phlebotomy, and was decreased when erythropoiesis was suppressed by hypertransfusion (16). These results agree well with the production rates of "early labeled" bilirubin in dogs with normal, increased, and decreased erythropoiesis (19).

3. The oxidation efficiency of Hopcalite was shown to be greater than 80% over the range of concentrations tested (Table 2). This range includes the CO concentrations normally found in small animals by Sjöstrand (8).

4. Numerous trials with 2 or more ethanolamine absorbers in series showed that all the activity was removed by the first absorber tube. The absorption capacity of the solution used is more than sufficient for 6-hr collections, the longest intervals routinely used.

The dose of 50 μCi was chosen so that the counting rate during the destruction of the labeled red blood cells would be at least twice the background counting rate. At the specific activities available (22 mCi/millimole), 50 μCi means a dose of approximately 0.17 mg of glycine. Since it has been estimated that the turnover of glycine is approximately 27 mg/hr in a 350-g rat (21), this dose is probably still in the "tracer" range.

E. Summary

A method is presented for the separation, detection, and quantitation of endogenously produced carbon-14 labeled carbon monoxide in the rat, following injection of glycine-2-^{14}C. In this method, respiratory $^{14}CO_2$, the only significant breath contaminant, is removed with a soda lime absorber. The remaining breath activity, due primarily, if not entirely, to ^{14}CO, is oxidized to $^{14}CO_2$ by Hopcalite, absorbed in an ethanolamine-containing solution, and counted by liquid scintillation. Standard ^{14}CO and $^{14}CO_2$ gases, as well as animal experimentation, confirm this method's ability to measure ^{14}CO and $^{14}CO_2$ production rates simultaneously, following a single injection of labeled glycine. Examples are given to show that this continuous, in vivo, and easily performed method can give important information concerning heme catabolism. The technique will be shown in the following sections to provide a unique source of information in the study of disease processes characterized by abnormal heme catabolism in man and animals.

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III. ENDOGENOUS PRODUCTION OF ^{14}CO: A METHOD FOR CALCULATION OF RBC LIFE SPAN DISTRIBUTION IN VIVO WITHOUT BLOOD SAMPLING (1)

A. Introduction

Considerable interest has centered upon the determination of RBC life span in normal and disease states in animals and man. Although some information concerning RBC life span can be obtained from studies of the reticulocyte count, daily fecal stercobilin excretion, and the endogenous production of nonlabeled CO, it is only with the use of tracers or isotopes that detailed information can be obtained for quantitative definition of the parameters describing the distribution of RBC life spans in the peripheral blood.

The previously available tracer methods for determination of these parameters have been adequately reviewed elsewhere (2-4). It has been stated that most of the methods in current use suffer from one or more defects (2). In Chapter II a method was introduced for the in vivo detection and quantitation of endogenously produced ^{14}CO in the breath of mammals, following injection of glycine-2-^{14}C (5). The number 2 (methylene) carbon atom of glycine is incorporated into the heme moiety of circulating RBC hemoglobin, and is the unique source of the 4 methene bridge carbon atoms of heme (6). When RBC are destroyed in the body, the heme ring is opened at the alpha-methene bridge carbon atom, with the quantitative oxidation of this carbon atom to CO (7, 8). Thus, 1 mole of CO is produced per mole of heme catabolized. Correspondingly, for each molecule of heme labeled with carbon-14 at the alpha-methene position, 1 molecule of ^{14}CO is produced. The ^{14}CO thus produced is excreted rapidly and quantitatively in the breath, without significant oxidation to $^{14}CO_2$ (9, 10). 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 accurately studied by this tracer method, without resort to venesection or collection of other body fluids.

In this chapter a kinetic analysis of RBC production and destruction will be presented, using analysis of ^{14}CO appearance in the

expired air following glycine-2-^{14}C administration in mice and rats having normal RBC survival patterns as well as representative disorders of RBC survival.

B. Materials and Methods

Animals used were 300-400 gram, male, specific pathogen-free buffalo rats (Simonsen Laboratory, Gilroy, California), and 17-26 gram female LAF_1, SEC/1 ReJ, and WC-B6 mice (courtesy of Drs. E. S. Russell and S. E. Bernstein, Jackson Laboratory, Bar Harbor, Maine). Rats were studied individually following intravenous injection of 50 μCi of glycine-2-^{14}C (specific activity 20-28 mCi/millimole; New England Nuclear Corp., Boston, Massachusetts) under light ether anesthesia. Each experiment in the mice was performed in groups of 2-5 animals, each simultaneously injected with 10 μCi of labeled glycine intravenously without anesthesia, and placed as a group into the collection system.

Labeled CO was detected and quantitated by the method described in Chapter II. Previous studies have shown that this continuous, in vivo method is an accurate means of measuring degradation of labeled heme(s) in intact animals (Landaw, Callahan, and Schmid, unpublished results), and that contamination by endogenously produced $^{14}CO_2$ and other volatile compounds in breath, urine, and feces is negligible (5).

Breath was collected from experimental animals over a period of time from 1-10 hr, with the animals breathing either room air or water-pumped compressed air, while enclosed in a 2-liter metabolism cage. Aliquots of the ethanolamine absorber solution were counted in duplicate in a liquid scintillation counter for a period of time sufficient to produce a counting error of less than 2% (95% confidence level). Samples were internally standardized with toluene-^{14}C for the determination of counting efficiency. Carbon monoxide production rates were stated in terms of dpm/hr of ^{14}CO. No attempt was made to determine total unlabeled CO production or to determine ^{14}CO specific activities.

Uptake of labeled glycine into heme and hemoglobin of circulating RBC was studied in normal buffalo rats and LAF_1 mice. Following injection of the labeled glycine, small aliquots of peripheral blood (less than 0.15 ml each) were removed via tail vein puncture of rats at varying times. The total amount of blood removed from each animal thus studied was always less than 5% of the calculated blood volume. For the study of labeled glycine incorporation into circulating RBC of mice, 1-2 animals were sacrificed at each time period. The RBC were washed 3 times in saline after the buffy coat was removed, and hemin was extracted and crystallized by the method of Labbe and Nishida (11). Hemin crystals were dissolved in pyridine, and hemin concentrations were determined spectrophotometrically. Hemin activity was determined by counting aliquots of the hemin solutions by liquid scintillation in a toluene, PPO solution, with suitable internal standardization and background correction for the color quenching caused by the hemin. Duplicate determinations of hemin specific activity (dpm/mg) agreed within 2%. No change from the initial specific activities was noted following one or two recrystallizations of the material.

In a few animals, uptake of glycine into RBC hemoglobin was studied by hemolysis of washed RBC in distilled water, and centrifugation of the hemolysate to remove RBC stroma. An aliquot of the supernatant hemoglobin solution was analyzed for HGB concentration spectrophotometrically, and duplicate aliquots were plated on aluminum planchettes at infinite thinness and counted on a gas-flow proportional counter. Uptake of the carbon-14 label into RBC hemoglobin and heme was analyzed using a visual best fit of appropriate mathematical form after plotting on semilogarithmic paper, or by using appropriate computer programs.

For the experiments involving cross-transfusion, male buffalo rat donors were prepared as follows: one group of rats was given 50-100 μCi of glycine-2-^{14}C intravenously, and sacrificed 24 hr later. A single rat was injected with 50 μCi of labeled glycine and sacrificed 14 days later. Blood was removed by aortic puncture, under deep ether

anesthesia, and washed three times in saline. The hematocrits were adjusted to 45-55% by the addition of saline following the final wash, and 2-3 cc of the RBC suspension was injected intravenously via a lateral tail vein into a normal compatible host.

A group of 5 LAF_1 mice were hypertransfused by intraperitoneal injection of packed RBC from isologous donors. Each mouse received 1 ml of packed cells (hematocrit 60-70%) intraperitoneally 29 and 28 days prior to injection of labeled glycine. Three days prior to administration of labeled glycine, RBC transfusions were again performed at the same dosage, at a time when the animals' hematocrits were in the range of 60-70%. Ten microcuries of glycine-2-^{14}C were then injected into each mouse intravenously (day 0), and breath collected for ^{14}CO production during the next 110 days. The hematocrit of each of the animals was maintained in the range of 55% or above for most of this time by further injections of packed RBC on the 22nd, 39th, and 44th day after the injection of the labeled glycine.

Red blood cell survival studies were performed in 5 adult rats with experimentally induced anemia, and in an additional normal rat 39 days following splenectomy. One male rat of the Sprague-Dawley stain developed iron deficiency anemia shortly after total gastrectomy.* The anemia responded to parenteral iron dextran therapy (Imferon, Lakeside Laboratories, Inc., Milwaukee, Wisconsin), and over the next 12 months the animal's blood picture slowly reverted to the original picture of a severe hypochromic anemia. This animal was studied when the anemia was stabilized at a hemoglobin level of approximately 3 grams %. Three male buffalo rats were given 4 mg of phenylhydrazine per 100 grams of body weight per day subcutaneously on days 0, 1, and 2. One rat was then injected with 50 μCi of glycine-2-^{14}C 3, 7, or 11 days following the third dose of phenylhydrazine. A male buffalo rat was splenectomized 39 days prior to injection of 50 μCi of labeled glycine. At the time of labeled glycine administration the hematocrit and reticulocyte counts had returned to preoperative

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*Kindly provided by Dr. Samuel Lepkovsky, Dept. of Poultry Husbandry, University of California, Berkeley.

levels. Two male buffalo rats were injected with 50 μCi of labeled glycine 106 days following trans-oral hypophysectomy. Due to an inadequate diet, these animals lost weight continuously during the period of study and died approximately 75 days after glycine injection.

All data for ^{14}CO 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 (12). 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 Corporation CDC-6600 digital computer. Copies of this program are available upon request. The results of RBC survival obtained from such programs were stated in terms of the following parameters:

- k rate of random hemolysis (%/day)
- T mean potential RBC life span (days)
- σ spread of life spans about the mean potential life spans (days)
- C percent of injected glycine incorporated into RBC heme (%)
- S percent of labeled RBC dying of senescent processes (%)
- $\overline{T}$ mean overall RBC life span (days)
- $(1/\lambda)$ mean RBC heme labeling time (days).

In addition, the contribution to ^{14}CO production arising 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 exponential terms. A complete discussion of the derivation and use of the equations developed for the determination of these parameters can be found in the Appendix.

Standard statistical tests were used to compare the resulting parameters of RBC survival in various treatment groups with those obtained in normal, control animals. Results were expressed in terms of "p" (Student's "t" test). Values for "p" of less than 0.05 were considered statistically significant.

C. Results

Figure 1 demonstrates the pattern of ^{14}CO appearance in the breath of mice given a single injection of glycine-2-^{14}C in the presence and absence of erythropoiesis. In this experiment erythropoiesis was completely suppressed in one group of LAF_1 mice by hypertransfusion (closed circles). The peak in ^{14}CO excretion seen in the normal mice (open circles) at about 53 days ("late peak") is absent in the hypertransfused mice, and is identified with destruction of circulating labeled RBC. There is considerable ^{14}CO excretion during the first few days following labeled glycine administration in both groups of mice, identifying at least a part of this "early labeled peak" (ELP) of ^{14}CO production with the catabolism of nonhemoglobin hemes (13-17). Production of labeled CO in the hypertransfused mice continues at a low, but measurable, level from the end of the downslope of this ELP to more than 110 days after injection of the labeled glycine. The production of ^{14}CO by these mice during the entire duration of ^{14}CO collection (3 to 110 days following glycine-2-^{14}C) could be described by the sum of two exponential terms, one with a half-time of 2.7 days, and the other with a half-time of 52 days. Similarly, ^{14}CO production in starved, hypophysectomized buffalo rats, in which erythropoiesis was also greatly depressed (18), could be described by the sum of two exponential terms, with a half-time of 2.5 and 100 days (Fig. 2). The presence of two similar components can also be detected in the curve of ^{14}CO excretion in the normal mice (Fig. 1).

Labeled CO excretion was measured in buffalo rats following cross-transfusion of labeled cells from donors given glycine-2-^{14}C 1 day or 14 days (Fig. 3) prior to the cross-transfusion. Labeled CO production virtually ceased following the "late peak" in these animals. In such studies there is no evidence for the second, or slower exponential process seen in the normal or hypertransfused mice, which identifies this slower component with processes other than those associated with the destruction of the initial cohort of labeled hemoglobin heme from circulating RBC.

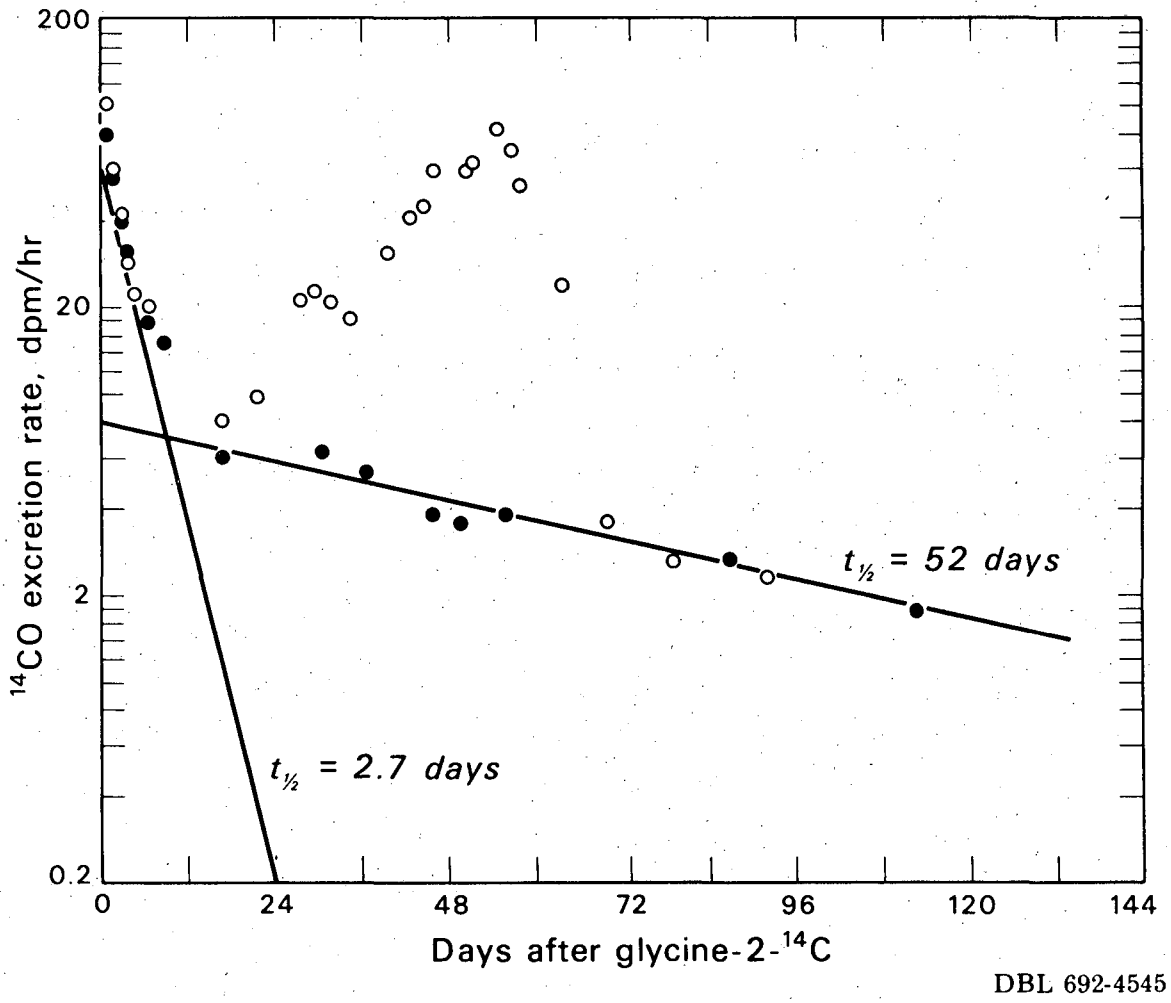

Fig. 1. Endogenous ^{14}CO excretion rate in groups of normal and hypertransfused LAF_1 mice. This graph shows a semi-logarithmic plot of ^{14}CO excretion rate (ordinate, dpm/hr of ^{14}CO) versus time after injection of glycine-2-^{14}C abscissa, days) in a group of 5 normal LAF_1 mice (open circles) and a group of 4 hypertransfused LAF_1 mice (closed circles). The solid lines represent the least-squares best fit of the data points to Eq. 6. The half-times for the two exponential components are given on the figure.

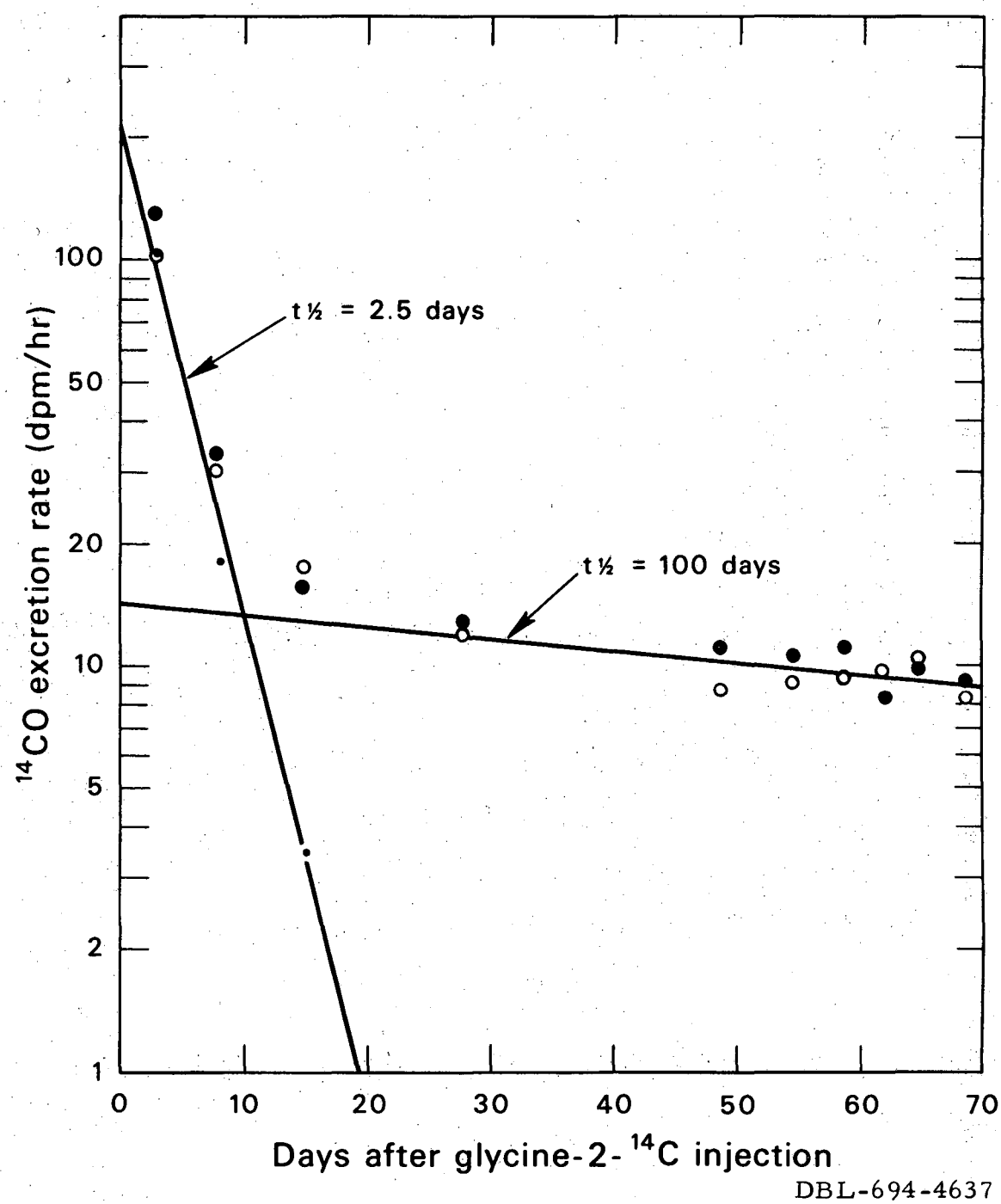

Fig. 2. Endogenous ^{14}CO excretion rate in two starved hypophysectomized rats. The ordinate and abscissa are the same as for Fig. 1. Rats were injected with 50 μCi each of glycine-2-^{14}C 106 days after hypophysectomy. Data points obtained from each animal are indicated by open or closed circles. The solid line represents the least-squares best fit of the averaged data points to Eq. 6. The half-times for the two exponential components are given on the figure.

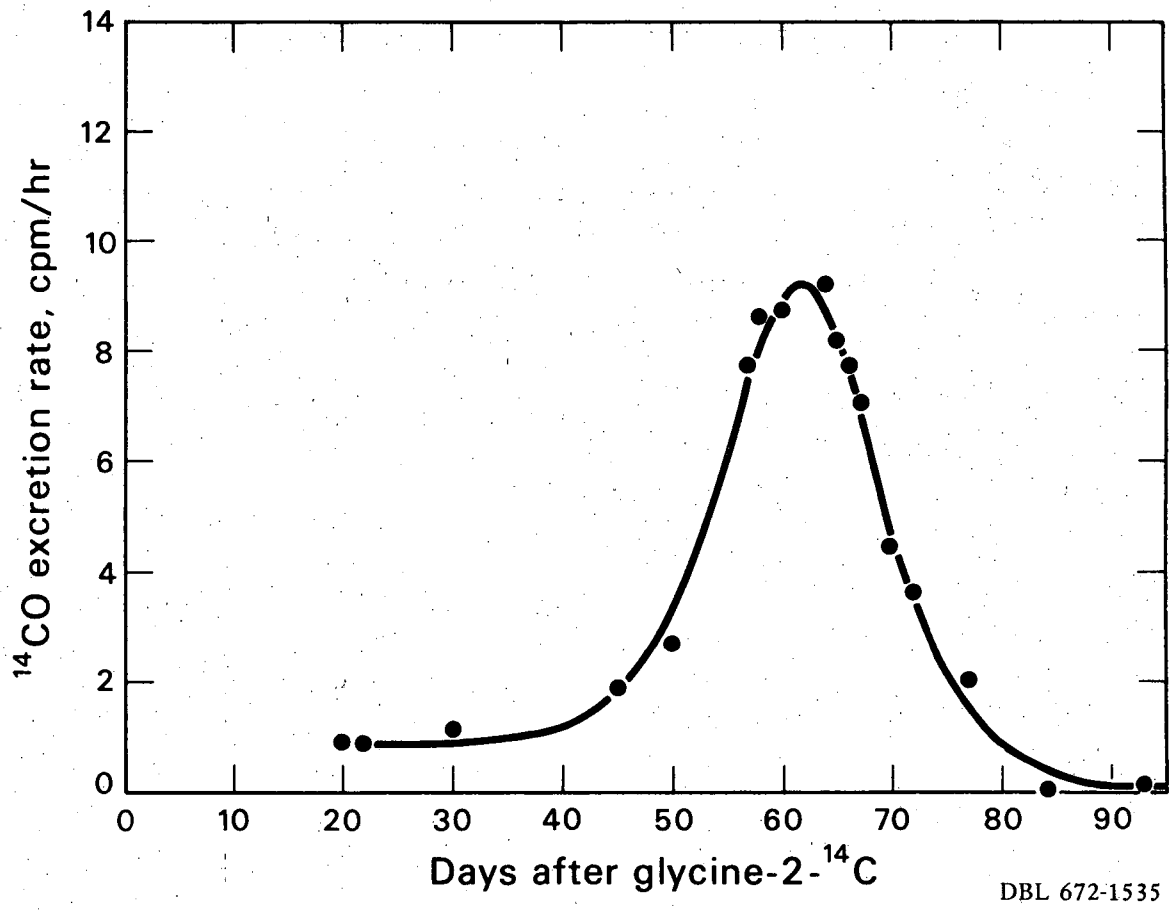

Fig. 3. Endogenous ^{14}CO excretion rate in a normal host rat transfused with a cohort of labeled RBC from a normal donor. The ordinate and abscissa are the same as for Fig. 1. The glycine-2-^{14}C was injected into the donor at time zero. Red blood cells were collected and transfused into the host on day 14, and ^{14}CO collection began on day 20. The data points are shown as closed circles, while the solid line is the least-squares best fit of the data points to Eq. 2.

Figures 4 and 5 present the rate of ^{14}CO excretion from a normal buffalo rat and a group of 5 normal LAF_1 mice, respectively, following a single injection of glycine-2-^{14}C. In both figures, data points are shown as closed circles, with the least-squares best fit of these data points to the proposed function describing the rate of ^{14}CO excretion shown as a solid line (Eq. 7, Appendix). The ^{14}CO excretion rate arising from sources other than the degradation of the initially labeled cohort of RBC is shown as the coarsely dashed line in these figures, and is obtained from the last two terms of Eq. 7. The rate of ^{14}CO excretion representing destruction of the initial cohort of labeled RBC is shown as the finely dashed line, and is obtained from the first term of Eq. 7. These curves define the time at which the maximum rate of ^{14}CO excretion is seen as approximately 65 days in the buffalo rat and 53 days in the LAF_1 mouse.

Figures 6, 7, and 8 present results obtained in three rats with experimentally induced alterations in erythropoiesis. One animal had anemia induced by daily injections of phenylhydrazine 5, 4, and 3 days prior to glycine injection (Fig. 6), and the second had severe, chronic iron deficiency anemia (Fig. 7). The third rat (Fig. 8) was studied 39 days following splenectomy. Blood values obtained on the day of glycine injection for each of the three animals are shown in Table 1 along with the results for the parameters of RBC survival in the same animals.

The three animals with phenylhydrazine-induced anemia showed marked increases in ^{14}CO production from 3 to 9 times normal, with a well-defined "late peak" centered around 50 (Fig. 6), 56, and 62 days, respectively, in contrast to normal rats which show a peak around 65-68 days. The general shape of the curves did not otherwise appear to be appreciably altered from that of the normal rats. In the iron deficient rat (Fig. 7), the virtual absence of the "late peak" should be noted, the remnants of which can be appreciated as a shoulder in the curve at around 40-50 days. The "corrected" CO curve may be adequately approximated by a single exponential function for the time

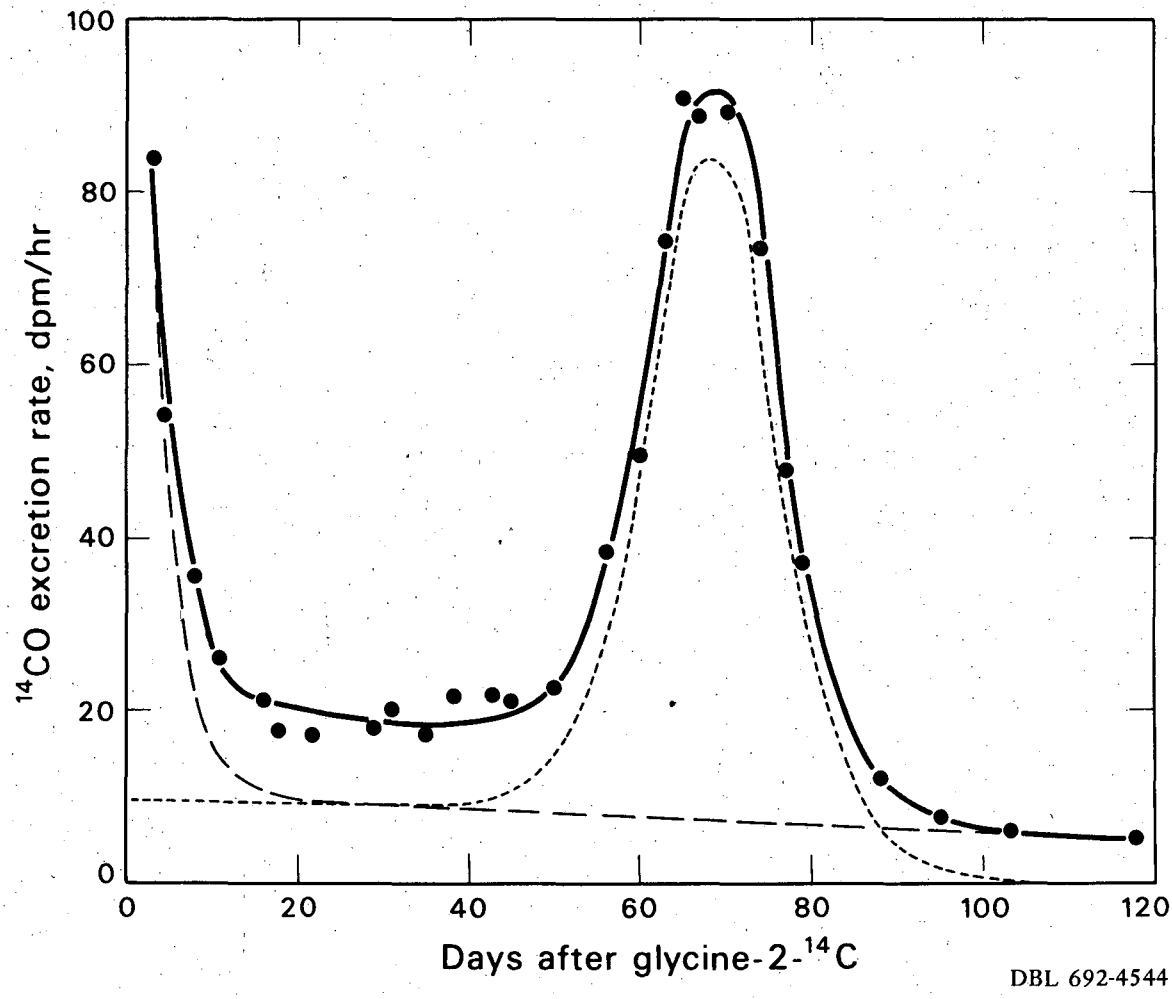

Fig. 4. Endogenous ^{14}CO excretion in a normal buffalo rat. Ordinate and abscissa same as in Fig. 1. Data points represented as solid circles. The solid line represents the least-squares best fit of Eq. 7 to the data points (accounting for all sources of ^{14}CO production). The finely dashed line represents the calculated production of ^{14}CO from the destruction of the cohort of labeled RBC (Eq. 2), while the coarsely dashed line represents the calculated production of ^{14}CO from destruction of labeled nonhemoglobin hemes (rapid component, Eq. 6) as well as recycling of labeled glycine (slow component, Eq. 6).

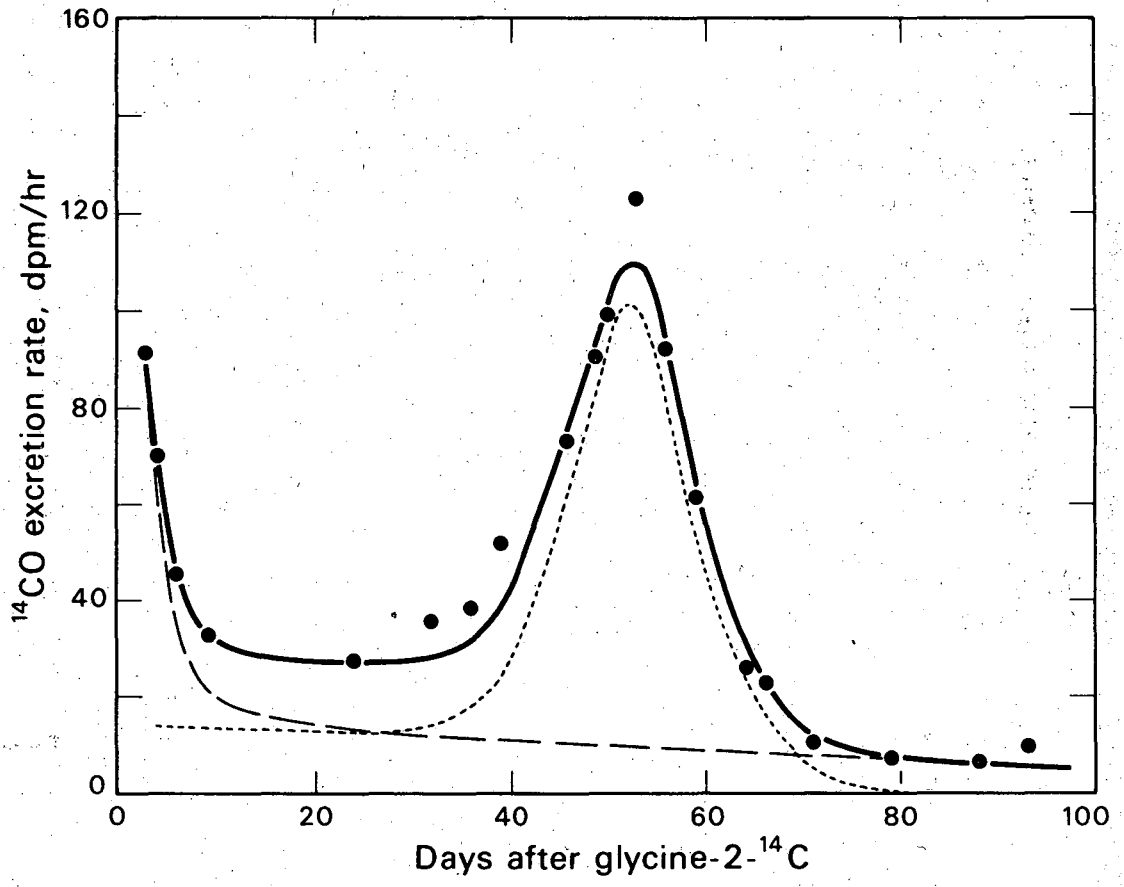

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Fig. 5. Endogenous ^{14}CO production in a group of 5 normal simultaneously injected LAF_1 mice. Description same as for Fig. 3.

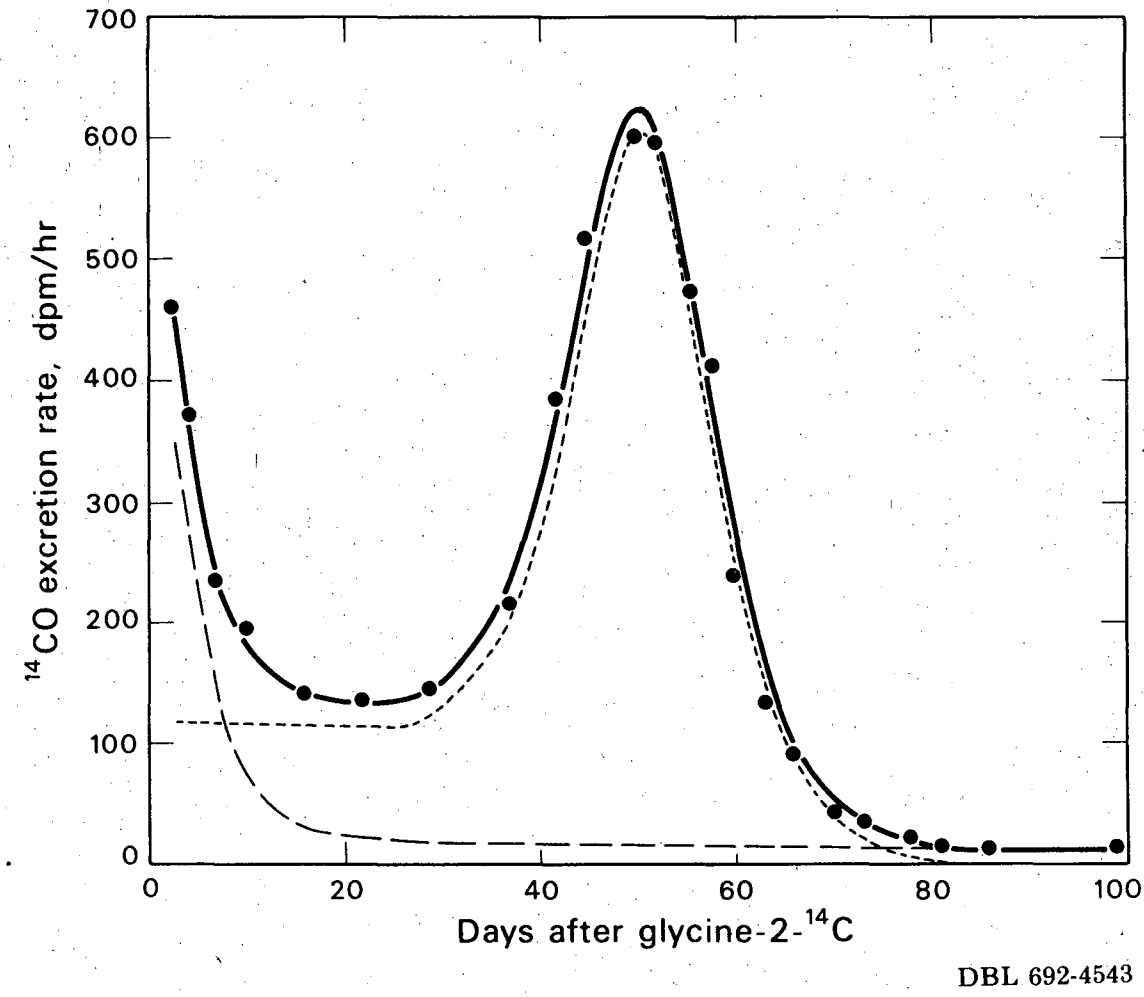

Fig. 6. Endogenous ^{14}CO excretion in a buffalo rat recovering from phenylhydrazine-induced anemia. Description same as for Fig. 3.

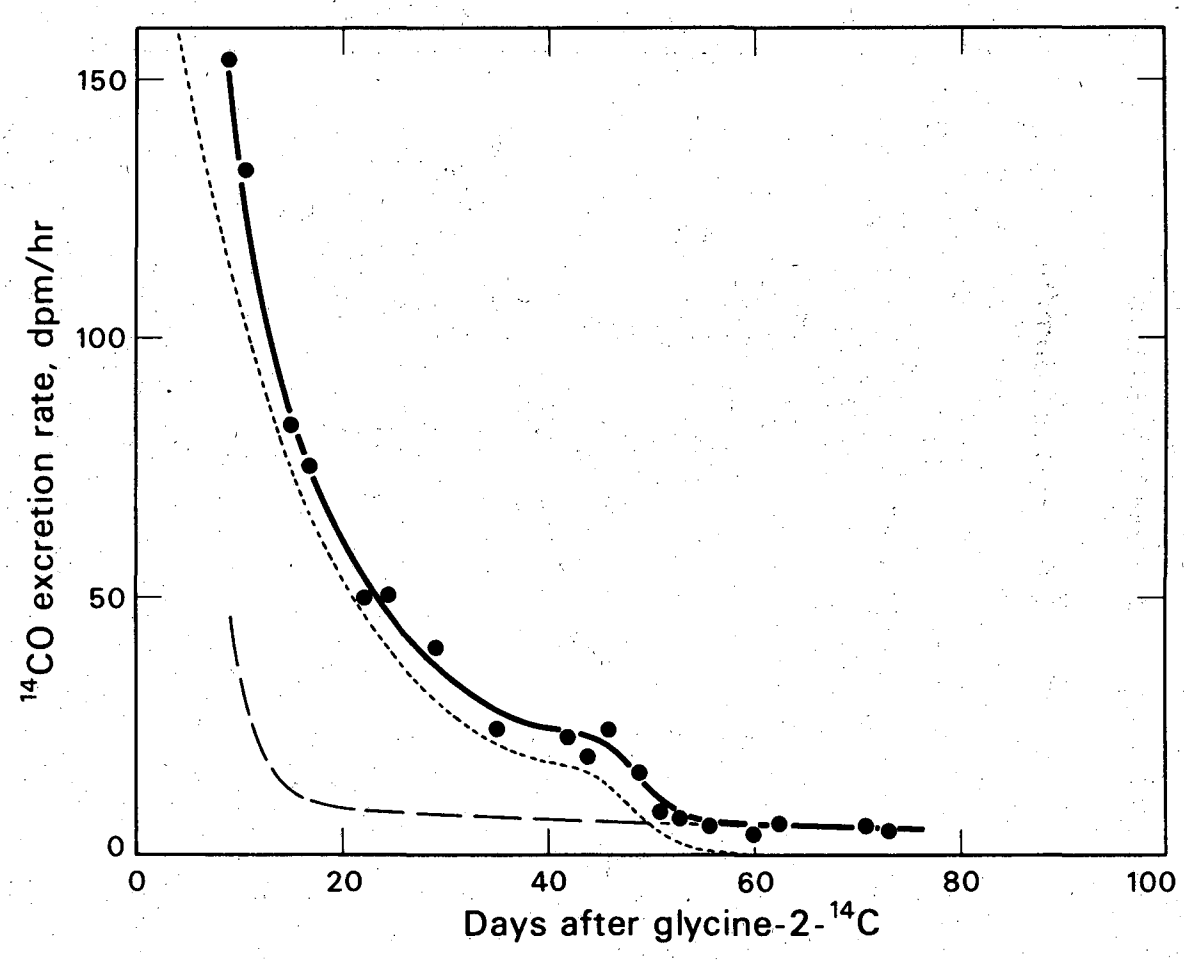

Fig. 7. Endogenous ^{14}CO excretion in a Sprague-Dawley rat with iron deficiency secondary to gastrectomy. Description same as for Fig. 3.

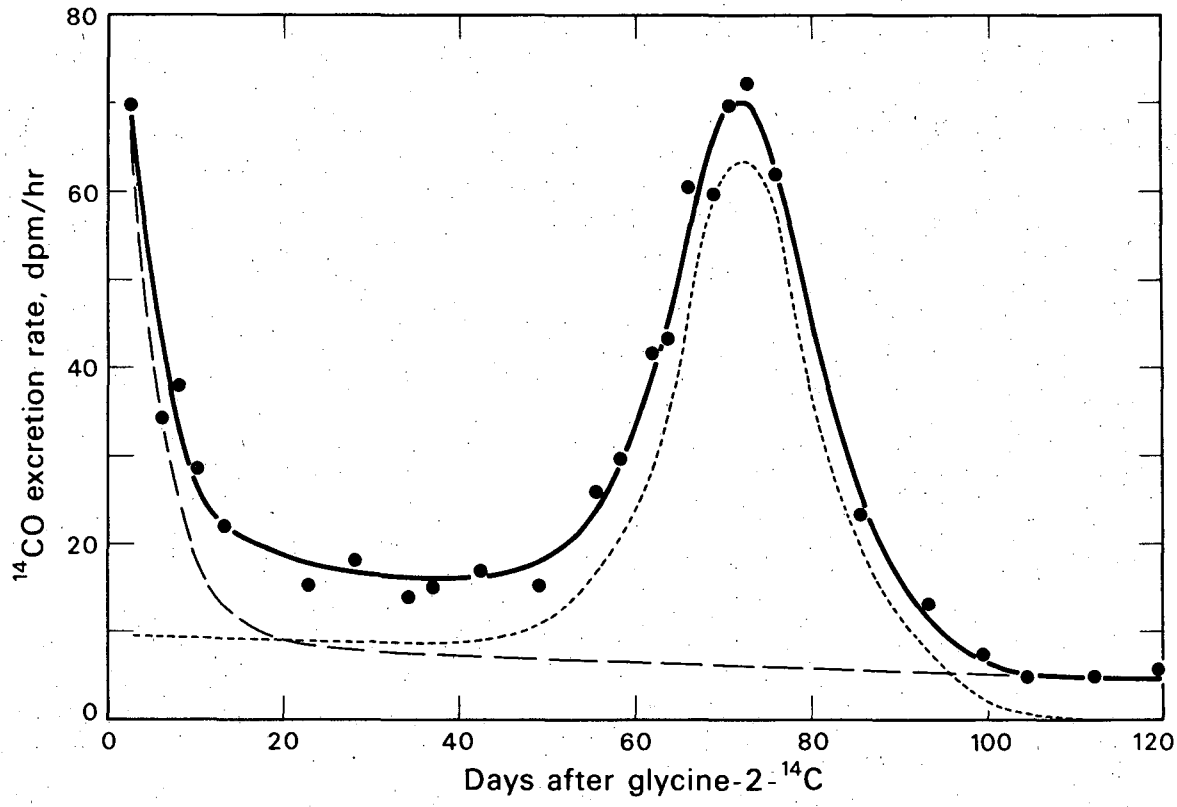

Fig. 8. Endogenous ^{14}CO excretion in a splenectomized buffalo rat. Description same as for Fig. 3.

Table 1. Blood values and parameters of RBC survival in normal and experimental rats.

Parameter	Average or range of 6 normal buffalo rats (mean ± S.E.) (Fig. 4)	Average of 6 cross transfused male buffalo rats (Fig. 3)	Anemia due to phenylhydrazine (Fig. 6)			Iron deficiency anemia (Fig. 7)	Buffalo rat post splenectomy (Fig. 8)
STANDARD HEMATOLOGIC TESTS							
Hemoglobin (grams %)	13.5 - 14.7 (range)	--	--	--	--	2.6	14.2
Hematocrit (%)	44 - 50 (range)	--	31.5	43.0	48.0	12.9	44.7
Mean corpuscular HGB concentration (grams %)	29 - 32 (range)	--	--	--	--	20	32
Reticulocytes (%)	1.5 - 3.5 (range)	--	80	64	20	32	2.3
PARAMETERS OF RBC SURVIVAL							
Rate of random hemolysis (%/day) (k)	0.67 ±0.07	0.60 ±0.05	0.79	0.38	0.35	7.02[b]	0.52
Corrected mean potential lifespan (days)(T')	66.2 ±0.7	63.0 ±0.7[a]	50.1[b]	56.0[a]	61.7[a]	44.4[b]	71.8[a]
Standard deviation about mean potential life span (days) (sigma)	7.6 ±0.6	8.6 ±0.4	8.1	7.1	9.4	5.6	9.3
Percent uptake of glycine into RBC heme (%) (C)	0.247±0.032	--	2.230[b]	1.444[b]	0.781[b]	0.517[a]	0.331
Mean overall RBC life span (days) ($\overline{T}$)	54.5 ±1.5	53.3 ±0.6	42.2[a]	51.3	56.2	14.5[b]	60.6
Percent of RBC death by senescence (%) (S)	64.8 ±2.7	69.0 ±2.7	67.6	81.1	80.5	4.9[b]	68.8

[a] $p < 0.05$.

[b] $p < 0.001$.

interval between 7 and 40 days following glycine injection, with a half-time of approximately 10 days, corresponding to a rate of random hemolysis of approximately 7 %/day, or about 10 times the normal rate. In the splenectomized rat (Fig. 8) the only alteration from normal is a "late peak" centered about 72 days, approximately 6 days greater than the average for the normal rats.

Table 1 summarizes the hematologic finding and the results of the parameters of RBC survival obtained from endogenous ^{14}CO excretion in 6 normal rats, as well as the rat with iron deficiency anemia, and the splenectomized rat. Also included in this table are the average values for these parameters in six cross-transfused rats, one of which is illustrated in Fig. 3. In these rats, the only significant alteration from normal is a mean potential life span approximately 3 days shorter than normal.

Table 2 summarizes the results of the parameters of RBC survival in 3 groups of LAF_1 mice, one group of SEC/1Re J mice, and 1 group of WC/B6 mice. Results for all parameters were essentially identical in the three separate strains except for the mean potential life span (T) which varied from 47 days in the SEC/1Re J strain to 57 days in the WC/B6 strain. Random hemolysis in all strains averaged from 0.32 to 0.60 %/day, not greatly different from the values seen in the normal buffalo rats (0.67 %/day).

The appearance of the carbon-14 label in the heme of circulating RBC hemoglobin was studied in 3 normal male buffalo rats and in a group of simultaneously injected female LAF_1 mice. The curves of RBC heme specific activity versus time after glycine injection were adequately fitted by a single exponential uptake function (Eq. 4, see Appendix), and yielded values for the mean labeling time of circulating RBC ($1/\lambda$) of 0.9 day for the buffalo rat and 1.1 days for the LAF_1 mice. These values for mean labeling time were used to correct the mean potential life span (T) for the delay in the appearance of the labeled RBC in the circulation (see Appendix).

Table 2. Parameters of RBC survival in three strains of normal mice.

Parameter	Mouse strain		
	LAF_1	SEC/1Re J	WC/B6
No. of animals studied	15	4	2
Rate of random hemolysis (%/day) (k)	0.60 ±0.17[a]	0.32	0.54
Corrected mean potential life span (days) (T')	51.8 ±0.4	47.1	57.3
Standard deviation about mean potential life span (days) (σ)	9.1 ±1.2	8.8	7.8
Percent uptake of glycine into RBC heme (%) (C)	0.323±0.003	0.299	0.411
Mean overall RBC life span (days) ($\overline{T}$)	45.6 ±1.6	44.8	50.3
Percent of RBC death by senescence (%) (S)	74.1 ±6.7	86.2	73.5

[a]Mean ± S. E.

Figure 9 shows the combined data for the specific activity of hemoglobin and/or hemoglobin heme in normal buffalo rats given glycine-2-^{14}C. The shaded area represents the range of heme specific activities to be expected from Eq. 1 (see Appendix) from the parameters derived from the fit of ^{14}CO data to Eq. 7. It can be seen that, for virtually all the data points collected, the heme and hemoglobin specific activities actually measured in the blood agree well with those predicted from the ^{14}CO excretion in the breath.

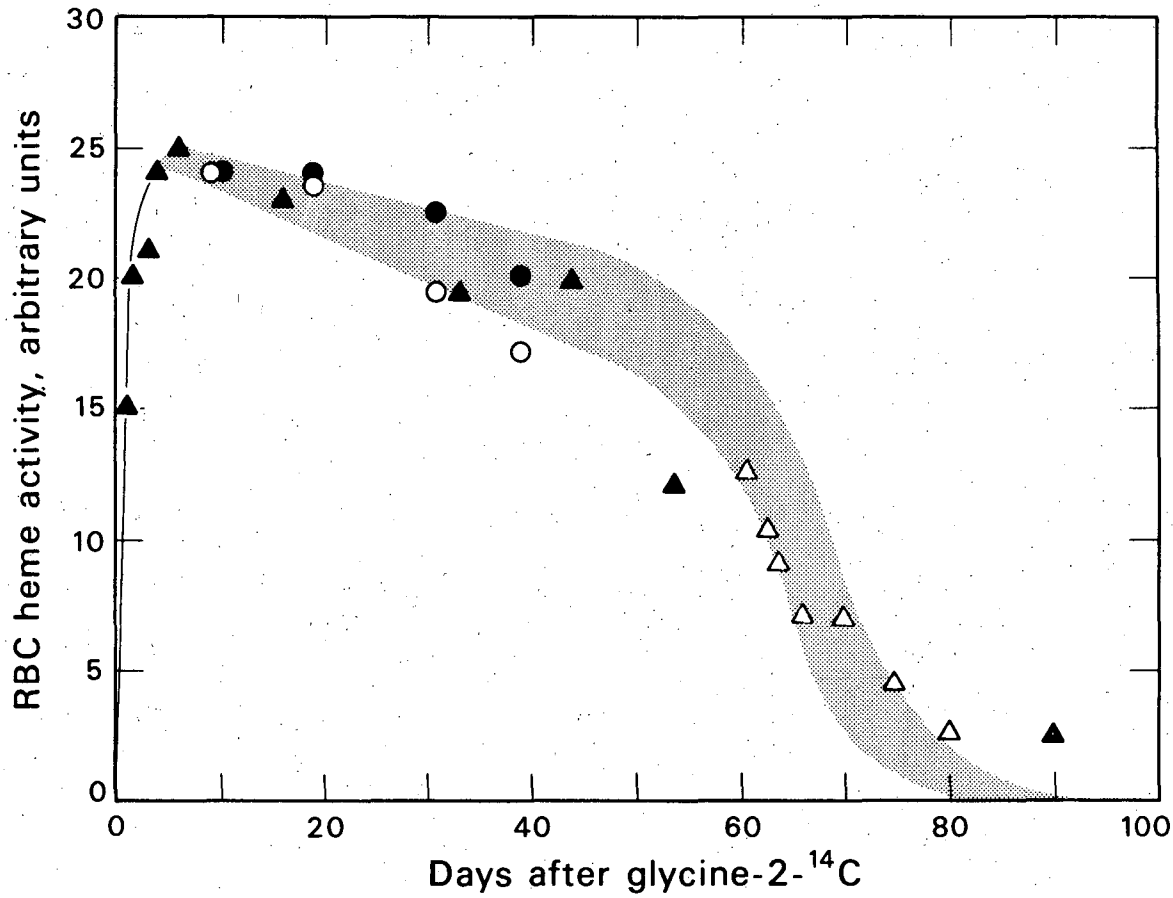

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Fig. 9. Comparison of measured circulating RBC heme ^{14}C specific activity with that calculated from ^{14}CO excretion in 6 normal buffalo rats. The ordinate represents normalized circulating RBC heme ^{14}C specific activity (arbitrary units), while the abscissa represents time after glycine-2-^{14}C injection (days). The shaded area represents the range of circulating RBC hemoglobin heme specific activity calculated from RBC survival parameters derived from ^{14}CO excretion rate data and Eq. 1. The 4 different symbols each represent measured heme or Hgb specific activity in a single normal buffalo rat.

D. Discussion

Experiments in the hypertransfused mice demonstrate that, following the third day after glycine-2-^{14}C injection, nonerythropoietic components of endogenous ^{14}CO can be described by the sum of two exponential terms. The first of these, with a half-time of 1/2 to 3 days, is identified with rapidly turning over heme compounds, such as have been postulated by others (19-21). The slower of these two components, with a half-time of about 52 days in the LAF_1 mouse and 100 days in the buffalo rat, probably represents turnover of glycine-containing structural proteins which release their glycine into the free glycine pool, from whence a portion is incorporated into rapidly catabolized hemes leading to production of labeled CO. The magnitude of this latter component was seen to be virtually independent of the state of erythropoiesis in rats and mice given glycine-2-^{14}C. A kinetics model consistent with these results is shown in Fig. 10. It should be mentioned that, prior to the third day following glycine injection, at least two additional exponential components of ^{14}CO production are noted, with half-times of less than 12 hr. These components comprise the "early labeled peak" of CO production, which is outside the scope of this paper, and which will be discussed in future communications. Due to their extremely short half-times, these components do not contribute significantly to ^{14}CO production following the third post-injection day, and have been ignored in this simplified model.

After the first week following injection of labeled glycine only the second, or slower, exponential process is responsible for significant production of ^{14}CO from sources other than degradation of circulating hemoglobin heme. An error in the estimation of the parameters describing this exponential process (B and k2, see Appendix) will therefore influence the value obtained for the rate of random hemolysis (k) and the size of the cohort (C) and the derived parameters of mean overall lifespan ($\bar{T}$) and percent senescent death (S). It was our experience that when the amplitude (B) and slope (k2) of this nonerythropoietic source of ^{14}CO were allowed to assume the full range of values which

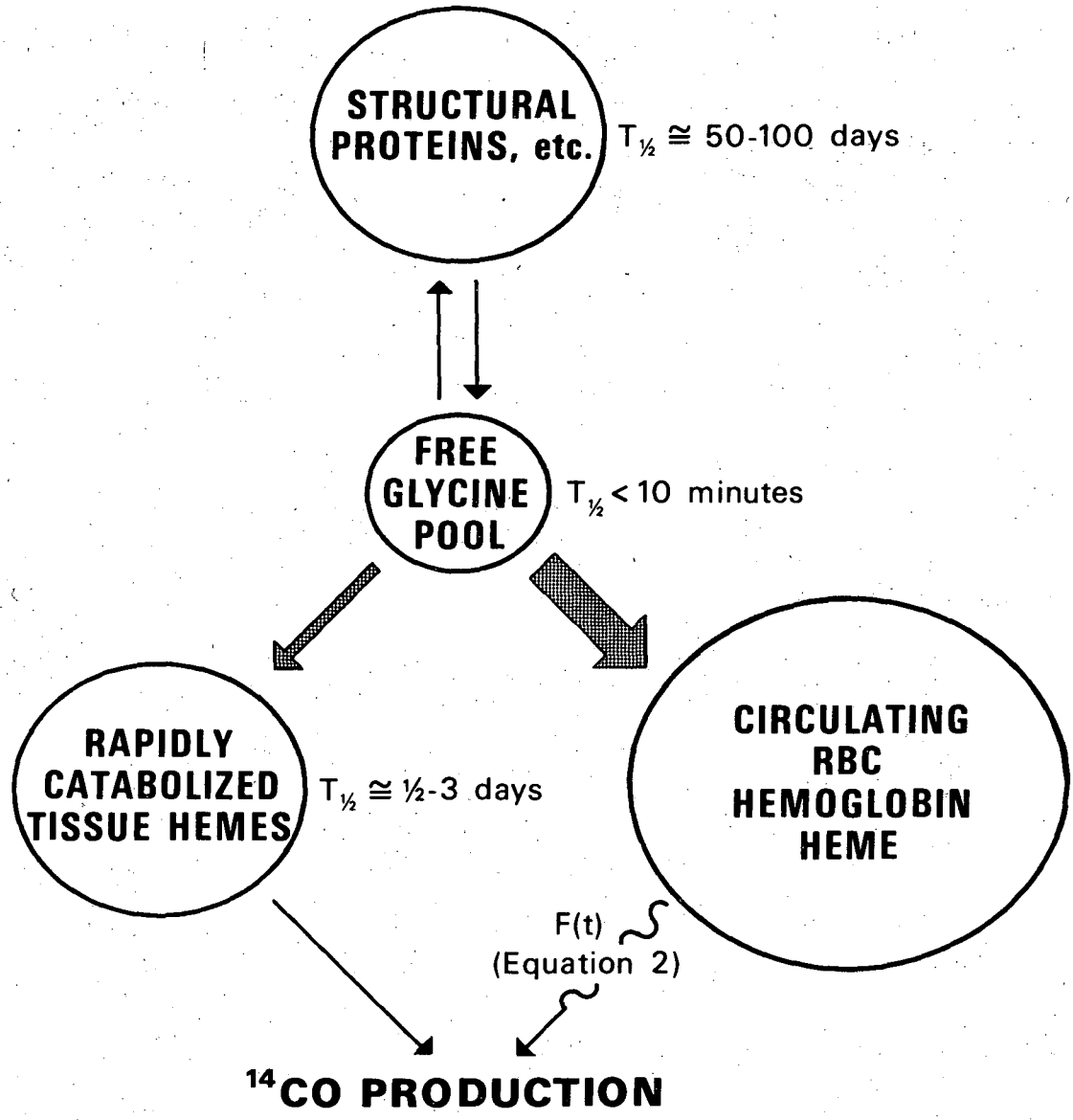

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Fig. 10. Proposed model for kinetics of ^{14}CO production following injection of labeled glycine in experimental and normal animals. (See Discussion for details.) This model is compatible with the data obtained in normal, hypertransfused, and cross-transfused animals, as well as data obtained in animals with experimentally induced alterations in erythropoiesis (for time after glycine injection greater than 3 days).

did not significantly alter the value of chi-square in the least-squares fitting program for Eq. 7 to ^{14}CO data, the parameter k was found to vary by no more than 0.20 %/day. Similarly, such "possible" variation in the parameter C was no greater than 10% of its calculated value, while that in the parameters T, σ, $\overline{T}$, and S was less than 3% of the calculated value. Such "possible" variations in these parameters become appreciably less as the erythropoietic rate increases, (as may be appreciated from inspection of Fig. 6) because of relative increase in the component of endogenous ^{14}CO production arising from catabolism of circulating hemoglobin heme.

The average value for random hemolytic rate (k) in 6 normal male buffalo rats of 0.67 %/day did not differ significantly ($p > 0.50$) from the average value obtained in 6 cross-transfused rats (0.60 %/day) in which no correction was necessary for ^{14}CO production unassociated with erythropoiesis. Results presented by Belcher and Harriss for normal rats, using ^{59}Fe labeling of RBC, yielded values for the rate of random hemolysis (k) of 0.22 to 0.69 %/day (22), in agreement with values obtained in the present study. Their values for the spread of life spans about the mean potential life span (α values of 0.20 to 0.46, corresponding to σ values of 3.7 to 8.5 days) are also in agreement with those presented here, but the values for the mean potential life span (T) using the ^{59}Fe technique (22-24) of 59 to 62 days are shorter than those obtained with the hemin-^{14}C method [68 days, (25)], and well outside of the range obtained in normal buffalo rats in the present study (65 to 69 days). In addition, the shorter mean potential life span noted with the ^{59}Fe method has been confirmed in studies performed in this laboratory using male buffalo rats, in which a mean potential life span of 59.6 days was found by the ^{59}Fe method, or approximately 6 days shorter than the value obtained in the same rat strain using the ^{14}CO method. This shortening of RBC survival probably reflects toxic alterations due to the multiple injections of iron dextran (22, unpublished observations of Shapiro, Landaw, Winchell, and Williams).

The value for the fraction of administered labeled glycine incorporated into circulating hemoglobin heme (C) obtained in the present study from measurement of breath ^{14}CO excretion alone (0.15 to 0.38%) agrees roughly with results obtained in this laboratory from direct study of incorporation of glycine into RBC hemoglobin heme [0.403 ± 0.039 (S.E.)%] and with that obtained by Robinson et al. (26) in the Gunn rat (0.62 ± 0.23 %). The reproducibility of this ^{14}CO method is attested to by the closeness between the results reported for 6 normal male buffalo rats from this laboratory and results obtained by cooperating investigators at the Ames Research Center, Moffett Field, California in 4 normal male buffalo rats obtained from the same supplier (k: 0.69, T': 68.3, σ: 6.4, C: 0.265, $\overline{T}$: 55.3, and S: 62.5; S. A. Landaw, and H. A. Leon, preliminary results).

As was suggested by Eadie and Brown (3) a complete study of RBC survival should evaluate senescence and random hemolysis separately, giving for the former an estimate of the mean potential life span and the degree of spread around it, and for the latter an estimate of the fraction of cells destroyed daily. The results shown in Figs. 6-8 and in Tables 1 and 2 demonstrate such analysis by this method in states in which one or more of these parameters may be altered. Such applications have already provided useful information (27), and further applications will be the subject of future communications. Since each of the groups included in Table 1 yielded a different pattern of RBC survival by the above criteria, they will be discussed in some depth in the following paragraphs.

An example of isolated shortening in the mean potential life span (T) without alteration in the rate of random hemolysis (k) was seen in the 3 rats recovering from phenylhydrazine-induced anemia (Fig. 6 and Table 1). In these animals, results indicate a shortening of overall RBC survival due solely to a shortening of the mean potential life span. It can also be seen in these 3 rats, that the mean potential life span was returning towards normal as the hematocrit, reticulocyte count, hemoglobin, and fractional glycine incorporation were returning to normal. These results, which have been confirmed in other rats so treated (27),

differs from those previously reported by Stohlman for similarly treated rats (23), in which the phase of senescence was not specifically studied, but which were reported to show increased rates of random hemolysis. Because of the limitations of the methods used in these previous investigations, the studies had to be performed in groups of animals considered comparable, with subsequent analysis of composite data. Since these animals are recovering from the induced anemia, and therefore have a constantly increasing RBC mass during a good part of the experiment, blood volumes must be taken for each data point, in order to correct for dilution of the tracer due to expansion of its pool size. Such corrections are not necessary in the cohort method presented in this paper, since only the total production of labeled CO is measured following the production of a cohort of labeled cells. Thus, RBC survival can be studied in individual animals, without correction for expansion (or contraction) of circulating blood volume.

An example of markedly increased rate of random hemolysis together with marked shortening of the mean potential life span is seen in the rat with post-gastrectomy iron deficiency anemia (Fig. 7). A shortening of overall RBC survival in iron deficiency in man (28) has been reported, while certain reports indicate a shortening of mean potential life span (29), and others can be interpreted as evidence of an increased rate of random hemolysis (30). The combination of markedly increased random hemolysis (10 times normal) plus a marked (33%) shortening of mean potential life span, which was seen in the animal reported herein, has not been previously documented. Despite the fact that only 5% of the labeled cells survived the phase of random hemolysis to die of senescent processes (Table 1), the ^{14}CO method was able to determine that there was also a markedly shortened mean potential life span. The reappearance of anemia in this animal is suggestive of continual blood loss, which might in itself cause a picture of increased random hemolysis. It would not, however, explain the markedly shortened mean potential life span seen in this animal. 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. In this animal, the production of ^{14}CO was also abnormal during the first 3 days following glycine injection, and indicated a pattern of "ineffective erythropoiesis," suggesting that all aspects of erythropoiesis were abnormal in this animal. Since the pattern of "ineffective erythropoiesis," increased random hemolysis, and shortened mean potential life span has not been seen in a series of acutely or chronically phlebotomized rats (27), this strongly suggests that the changes were due to the iron deficiency and not related to any coexisting chronic blood loss.

While not specifically covered in this study, the pattern of ^{14}CO production during the time of the "early labeled peak for CO" (0 to 3 days after glycine injection) can give important information concerning the fate of RBC in the sites of production ("ineffective erythropoiesis"), as well as the fate of newly-emerging RBC ("splenic sequestration and destruction"). Such studies will be the subject of future communications.

An example of an isolated increase in the mean potential life span (T) following splenectomy is shown in Fig. 8. In this animal the mean potential life span of 72 days (9 % greater than normal) was the longest of more than 70 rats with normal or abnormal erythropoiesis studied by this method, and was statistically significant ($p < 0.05$). This increased potential life span did increase the mean overall life span to about 61 days [normal = 54.5 ± 1.5 (S. E.) days], although this increase was not statistically significant ($p > 0.10$). Belcher and Harriss had also reported that splenectomy increased the mean potential life span in their rats from about 60 days in the normal to about 65 to 69 days in splenectomized rats (22).

That minor alterations in RBC survival can be accurately defined is also exemplified in the results obtained in cross-transfused rats (Fig. 3, Table 1). Red blood survival in these rats did not differ significantly for any of the parameters of RBC survival in normal rats except for a 4.5 % shortening of mean potential life span from 66 to 63 days ($p < 0.05$). Such shortening of mean potential life span following cross-transfusion has been regularly noted in similarly-treated rats

in this laboratory for both normal and phenylhydrazine-treated cells, and very likely represents trauma imposed on the RBC from the handling, washing, and injection of these cells.

Previous determinations of RBC survival in mice have yielded conflicting results, with one study reporting that RBC death was due solely to random processes (31), while others seem to indicate the complete absence of random hemolysis, with all cells dying of senescence (32-34). The present study, as well as studies of RBC survival in more than 24 additional LAF_1 mice with rates of erythropoiesis increased up to 3 times normal following hypoxia or erythropoietin injection (27) indicate that the rate of random hemolysis in LAF_1 mice is approximately equal to that seen in the rat, with a mean potential life span ranging from 51 to 53 days, with a mean of 52 days. Due to the narrow range of experimental values found for the parameter T in the LAF_1 mice, the values of 47 and 57 days found in the SEC/1Re J and WC/B6 strains, respectively, must be considered as significantly different (Table 2). Some of the previously reported variation in the values for mean potential life span in the mouse (32-35) are likely to be due to genetically determined variations among the various inbred mouse strains. Care must therefore be exercised in comparing RBC survival in various mouse strains, since this variation was as much as 21% in the present study (SEC/1Re J vs WC/B6).

The preceding discussion has shown that determination of the excretion rate of ^{14}CO in the breath alone can give reliable information concerning RBC survival in normal and abnormal conditions in animals. Figure 9 demonstrates that this method can also reproduce the heme specific activity curves expected from such studies, without resorting to blood sampling. In this figure the shaded area represents the range of hemin-^{14}C activity curves to be expected in normal buffalo rats from Eq. 1 (see Appendix), using parameters obtained from the best fit of the ^{14}CO data to Eq. 7. The data points represent male and female buffalo rats in which the specific activity of either hemoglobin or hemoglobin heme was followed serially, following injection of glycine-2-^{14}C. It can be seen that the actual heme or hemoglobin specific

activity curves are entirely consistent with the parameters derived from the ^{14}CO method. However, the great advantages of the latter 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 can be applied easily to the study of animals breathing artificial atmospheres without removing the animals from their environment (36). Recently Coburn (37) has shown that similar "late peak" curves can be obtained in man, suggesting that this technique should be applicable to man.

E. Appendix: Mathematical Model

Mathematical treatment of heme activity curves has been adequately presented elsewhere (2-4, 38). The simplest formula, applied to senescent and random destruction of a cohort of labeled RBC produced following injection of a pulse label such as glycine-2-^{14}C or ^{59}Fe is derived from the Verhulst-Pearl growth curve (39) and has the form

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

where $F(t)$ is the activity of hemoglobin or hemoglobin heme at a time t following appearance of tracer in the circulation, C is the maximum activity attained, T is the mean potential life span for senescent death, k is the rate of random hemolysis, and (a) is the coefficient of uniformity of life spans about T (3). This model, as well as its application to ^{14}CO kinetics, assumes that the parameters C, k, a, and T are invariant throughout the life of the cohort of labeled RBC. Since the rate of production of ^{14}CO within the body and its rate of excretion in the breath are equal to 1/8 of the destruction rate of labeled heme (40), there is a simple mathematical relationship between heme activity curves and the rate of production of ^{14}CO. The latter is given by 1/8 of the first derivative with respect to time of the heme-^{14}C activity curve, denoted by $F'(t)$ or dF/dt. This is shown in Eq. 2, where the minus sign is introduced since the activity in ^{14}CO increases as the activity in heme decreases:

$$-F'(t) = -dF/dt = \left(\frac{1}{8}\right) \frac{C e^{-kt}[k + (k + a)\, e^{a(t-T)}]}{[1 + e^{a(t-T)}]^2} . \qquad (2)$$

Both the term (a) of Eqs. 1 and 2 and the sigma term of the Gaussian function are measures of the distribution of values about a mean. The standard deviation, σ, increases as the spread about a mean increases, while (a) decreases as the spread about a mean increases. Because of the marked similarity in graphical form between the first derivative of the Verhulst-Pearl function and the Gaussian function, it is possible to determine an approximate identity between (a) and σ. This was done by determining the area subtended by the Gaussian and Eq. (2) over varying intervals, and determining the relationship between (a) and σ such that the sum of the squares of the differences between the two functions at 1/2, 1, 1-1/2, 2, 2-1/2, 3, 3-1/2, and 4 σ units from the mean were minimized. This calculation yielded the following relationship:

$$(a) \times \sigma \approx 1.701. \qquad (3)$$

This relationship was shown to hold for all positive values of (a) and σ obeying the above relationship, and it is thus applicable to all distributional ranges. The close similarity between these two functions can be seen in Fig. 11. According to Eq. (3), 69% of the total area subtended by curves of the form of Eq. (2) lies between the values of $\pm 1\ \sigma$ unit, 94% between $\pm 2\ \sigma$ units, and 99% between $\pm 3\ \sigma$ units. The corresponding values for the Gaussian are 68%, 95%, and 99.7%. In the body of this paper, values for σ corresponding to the calculated values of (a) are presented, since this concept of "spreadedness" is more commonly used.

Equation 1 would not be expected to adequately describe the entire heme activity curve following injection of labeled glycine since it does not take into account the time taken for the labeled cells to appear in the circulation, or the continued availability of label in the glycine pool. Allowance for delayed appearance of the labeled cells into the circulation can be made by studying the early phase of glycine

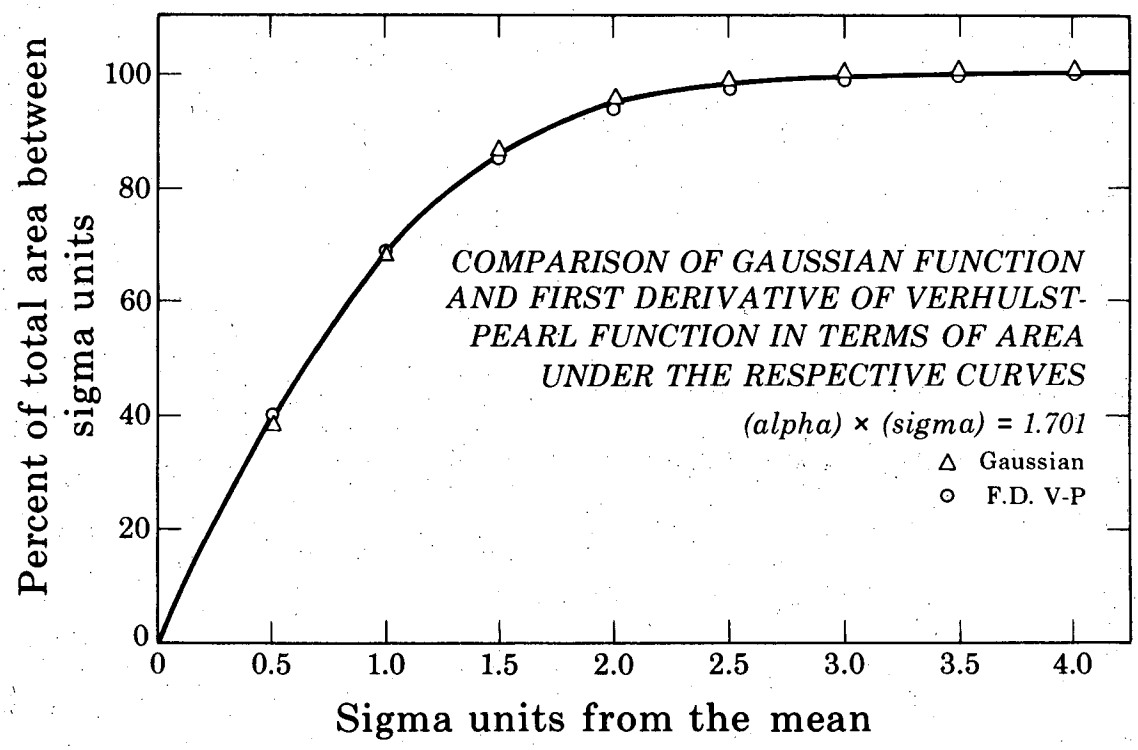

Fig. 11. Comparison of first derivative of Verhulst-Pearl function (F. D. V-P) (Equation 2 with k = 0) with a Gaussian distribution in terms of area under the curves between sigma units. Comparable values of sigma and alpha were obtained from Eq. 3. Note the extremely close agreement.

incorporation into heme of circulating RBC. As was shown by Shemin and Rittenberg for man (38), this phase can be adequately approximated by a single exponential uptake of the form

$$H(t) = C(1 - e^{-\lambda t}) \text{ (for } 14 \geq t > 0), \quad (4)$$

where H(t) is the activity of heme in circulating RBC. In the buffalo rats, λ averaged 1.1 per day, or a corresponding mean labeling time $(1/\lambda)$ of 0.9 day. In the LAF_1 mice, the average value for λ was 0.9 per day, or a corresponding mean labeling time of 1.1 days. The non-instantaneous appearance of label in the circulating RBC causes an error in the determination of the true mean potential RBC life span on the order of this mean labeling time. The corrected mean potential life span (T') can be approximated by

$$T' \approx T - 1/\lambda. \quad (5)$$

Although Shemin and Rittenberg introduced a more rigorously correct method for determination of the corrected mean potential life span (38, 41), in practice the values of T' obtained through the use of the formulae to be shown plus Eq. 5 when applied to data in mice and rats did not differ by more than 0.1% from the values obtained by using the more rigorous correction used in their paper, due to the large magnitude of the parameter λ in these animals (short mean labeling time).

Since the glycine does not act as a true "pulse" label, it may be argued that the values of (a) and σ are in error, since labeled cells enter the circulation over a prolonged period of time. In this case, (a) values would be too small (3), and σ values too large. We were able to estimate the limits of this error by comparing the values obtained for σ in the cross-transfused and the normal rats. In 5 rats transfused with "24-hour old" cells, σ averaged 8.7 days; in 1 rat transfused with "14-day old" cells, it was 8.1 days, and in 6 normal rats, in which labeled cells must have been entering the circulation continuously, it averaged 7.6 days. These values are not significantly different from each other, showing that, in the rat, the labeled cells actually do closely simulate a true cohort.

Berlin, Hewitt, and Lotz (42), Berlin, Waldmann, and Weissman (2), and Weissman, Tschudy, Bacchus, and Eubanks (43) supplied a

correction for the continuous availability of label in the glycine pool of the form

$$P(t) = \sum_{n=1}^{n} A_n e^{-k_n t} \qquad n = 1, 2, 3, \cdots. \qquad (6)$$

Results of study of urinary hippuric acid in human subjects given glycine labeled with carbon-14 or nitrogen-15 indicated that the continuous availability of label in the glycine pool causes a significant alteration in the kinetics of heme activity curves (42), and in any product for which glycine serves as a precursor (43). Berlin et al. (42) found two exponential terms in patients given glycine-2-^{14}C, while Weissman et al. (43) found three exponential terms following glycine-2-^{14}C and ^{15}N-labeled glycine for the same period of time. These results are similar to results presented here for ^{14}CO production in hypertransfused mice (Fig. 1) and starved hypophysectomized rats (Fig. 2), in which two exponential terms were apparent after the third day following administration of glycine, in the virtual absence of erythropoiesis. Similar curves have also been obtained in LAF_1 mice following 650 R of whole-body X-irradiation (unpublished results), and serve to define the precursor-product activity relationships during the length of the experiments in rats and mice. Similarly, these two exponential terms could be seen in all the animals injected with glycine-2-^{14}C with normal or increased rates of erythropoiesis (Figs. 1, 4-8), and were absent in rats transfused with washed labeled cells, in which there would be insignificant amounts of labeled glycine in the body during the length of the experiment (Fig. 3). Since the magnitude and halftimes of these two components were seen to be relatively independent of erythropoietic rate, it was concluded that two exponentials, comprising 4 separate parameters (A, k_1, B, k_2) were sufficient to account for this continued availability of labeled glycine throughout the period of study, following the third day after glycine injection, even though erythropoietic rate and heme turnover rates were not constant throughout this period of time. As mentioned earlier (Discussion) there are other exponential processes present before the third day, at a time when labeled cells are

still entering the circulation, but these processes do not significantly contribute to ^{14}CO production following the third post-injection day. When such exponential terms were added to Eq. 2, the following equation results, which is the one actually fit to the data:

$$CO(t) = \left(\frac{1}{8}\right) \frac{Ce^{-kt}\left[k + (k+a)\, e^{a(t-T)}\right]}{\left[1 + e^{a(t-T)}\right]^2} + A e^{-k_1 t} + B e^{-k_2 t} \qquad (\text{for } t \geq 3), \tag{7}$$

where A, B, k1, and k2 are appropriate constants. The first term of this equation represents the rate of ^{14}CO production due to the destruction of the initially labeled cohort of RBC, while the second and third terms represent those components of ^{14}CO production arising from other sources, such as the continuation of the processes responsible for the early labeled peak for CO (5, 37, 42) and those due to continued availability of label in the glycine pool.

The average survival time for circulating RBC (mean RBC life span, or $\overline{T}$) for both random hemolysis and senescence was determined from the following equation:

$$\overline{T} = \frac{8}{C} \int_0^\infty (t) \cdot F'(t)\, dt. \tag{8}$$

The rate of ^{14}CO appearance in the breath arising from random hemolysis is equal to the product: $k \times F(t)$, where $F(t)$ (Eq. 1) is the total ^{14}C activity in the alpha-methene position of circulating RBC hemoglobin heme of the initial cohort. Since the total amount of label appearing in the circulation in the alpha-methene bridge carbon atom of hemoglobin heme is equal to $\frac{C}{8}$, the percent of cells destroyed by random hemolysis (RH) is:

$$(RH) = \frac{800}{C} \int_0^\infty (k) \cdot F(t)\, dt \quad \text{and} \quad S = 100 - (RH), \tag{9}$$

where S is the percent of circulating RBC dying of senescent processes.

F. Summary

A new quantitative method 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-2-^{14}C. This method does not require sampling of blood or other body fluids, is independent of circulating blood volume or other steady state restrictions, and calculates 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 life span and spread of life spans about this mean can be calculated. Mean overall RBC life span 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 %/day, corrected mean potential life span 66.2 days, and standard deviation of life spans about this mean of 7.6 days. For normal female LAF_1 mice, average values were 0.60 %/day, 51.8 days, and 9.1 days, respectively. Results in two other inbred mouse strains were essentially similar save for a significantly shorter mean potential life span of 47.1 days in female SEC/1Re J mice, and a significantly longer mean potential life span of 57.3 days in male WC-B6 mice. Following splenectomy in the rat, an isolated, statistically significant increase in mean potential life span was seen. An isolated decrease in mean potential life span was presented for a rat recovering from phenylhydrazine-induced anemia. An example of a marked increase in random hemolysis together with a shortening of mean potential life span was presented in a rat with severe iron deficiency anemia.

The present method is shown to simultaneously determine all major parameters defining RBC survival, and as such, should be quite useful in the study of red cell disorders in animals and man.

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IV. THE EFFECTS OF GRADED ERYTHROPOIETIC STIMULATION ON RBC SURVIVAL IN THE MOUSE AND RAT AS STUDIED BY ENDOGENOUS ^{14}CO PRODUCTION

A. Introduction

Controversy exists with regard to the effect of increased erythropoietic rate on the survival of RBC. While certain previously published results indicate that increasing the rate of RBC production results in diminution in the survival of such cells in the circulation (1-6), other results indicate that increasing erythropoiesis has no effect on RBC survival (7-9). This question is by no means trivial since it has implications in such problems as the biochemical basis of RBC survival and in understanding the kinetic mechanisms involved when erythropoietic rate is increased.

In Chapter III a method was described for the determination of the distribution of circulating RBC survival times by measurement of ^{14}CO in the breath of animals following the injection of glycine-2-^{14}C (10). This continuous, quantitative, in vivo method obviates the necessity of obtaining any blood samples, and is independent of changes in total body RBC volume. It is the purpose of this chapter to present results using this method in the mouse and rat following graded erythropoietic stimulation by hypoxia, bleeding, erythropoietin (ESF) injection, or phenylhydrazine (PHH)-induced anemia.

B. Materials and Methods

Animals used for this study were female LAF_1 mice (Jackson Memorial Laboratory, Bar Harbor, Maine) and male buffalo rats (Simonsen Laboratory, Gilroy, California). The mice, weighing approximately 20 g each, were studied in groups of five animals, each mouse receiving 10 μCi of labeled glycine (specific activity: 20-28 millicuries per millimole; New England Nuclear Corp., Boston, Mass.) intravenously without anesthesia. The rats, weighing 300-350 g each, were studied individually, each rat receiving 50 μCi of labeled glycine intravenously under light ether anesthesia.

Labeled carbon monoxide was detected in the expired air of these animals by the method presented in Chapter II (11). A similar method has recently been introduced by White, Coburn, Williams, and co-workers for performing the same type of studies in man (12).

Production of ^{14}CO was divided into "early" and "late" phases by methods described in Chapters II and III (10, 11). The "early peak" was determined in one of two manners, as follows:

1) All of the ^{14}CO exhaled by the animal during the first 3-5 days following injection of the labeled glycine was measured. On subsequent days the quantity of exhaled ^{14}CO was measured for 5-hr periods. In this manner the exact magnitude and shape of the early peak could be accurately determined.

2) Labeled CO was collected at frequent 5-hr intervals during the period 3-18 days following injection of the labeled glycine.

In either method, the data points obtained after the third post-injection day could be approximated by a single exponential regression line, using a visual best-fit to the data points on semilogarithmic paper. The quantity of ^{14}CO excreted secondary to early peak processes was estimated in method 1 by adding the total activity obtained during the period of continuous collection of ^{14}CO to the calculated excretion from the end of the period of continuous collection to infinity, assuming a single exponential form to the ^{14}CO excretion data during this period. In method 2 the quantity of ^{14}CO excreted related to the early peak processes was estimated from the single exponential regression line alone. This was done by dividing the zero time extrapolate by the slope of the curve relating ^{14}CO excretion rate to time after injection of glycine-2-^{14}C. The second, slower exponential process, with a half-time of about 50 days in the mouse and 100 days in the rat (see Chapter III) was subtracted from all labeled CO production in these calculations of "early" and "late" peak fractionation, since this process does not appear to be associated with the destruction of the original cohort of labeled heme, but is probably due to persistence of label in the glycine pool.

After correction for the single exponential component due to continuation of the early peak, the late peak of ^{14}CO excretion was analyzed by fitting the corrected data obtained after the 20th day to appropriate mathematical functions (10, 13) utilizing a CDC 6600 digital computer. When thus applied to the data, this computer program provides the following parameters describing the distribution of RBC survival:

k = rate of random hemolysis (%/day)

T = mean potential RBC life span (days)

σ = standard deviation of life spans about the mean potential life span (days)

C = fractional incorporation of labeled glycine into circulating RBC heme (%).

Hematocrit determinations were performed using a semi-micro method. Hemoglobin concentrations and reticulocyte counts were performed using standard methods. The length of time for sampling the expired air ranged from 1-5 hr. Samples for liquid scintillation counting of the trapped ^{14}CO (as $^{14}CO_2$) were prepared in duplicate, and counted for a sufficient length of time so that the counting error would be less than 2%. Sample count rate (cpm) was converted to absolute disintegration rate (dpm) by the use of an internal standard of toluene-^{14}C (New England Nuclear Corp., Boston, Mass. and Nuclear-Chicago Corp., Des Plaines, Illinois). The maximum excretion rates for ^{14}CO obtained in this study were approximately 2000 dpm/hr during the early peak, and 500 dpm/hr during the late peak.

For the cross-transfusion experiments, donor cells labeled by glycine-2-^{14}C during the preceding 24 hr were removed via aortic puncture, and washed three times in isotonic saline. The hematocrits were then adjusted to 45-55% by the addition of normal saline after the final wash, and the cells were transfused intravenously via a lateral tail vein into normal compatible hosts.

Phenylhydrazine solutions were made fresh daily, and injected in a dose of 4 mg per 100 g of body weight intraperitoneally on days 0, 1, and 2. Labeled glycine was then injected into rats 2, 3, 4, 5, 7, or 11

days following the last injection of the phenylhydrazine. A complete early peak determination was made in the rats given labeled glycine 3 or 5 days following the last injection of phenylhydrazine (method 1). In all the rats given this drug, the early peak magnitude was also estimated by method 2.

Rats were bled one-third of their calculated blood volume, or 1.5% of their body weight (14) each day for three consecutive days, either by cardiac puncture or tail vein puncture, under light ether anesthesia. In an effort to avoid the production of lowered levels of serum iron as a direct result of the repeated bleeding, injections of iron dextran (Imferon, Lakeside Laboratories, Inc., Milwaukee, Wisconsin) were given subcutaneously on the day preceding each phlebotomy, for a total dose of 15-20 mg of elemental iron per rat. This is approximately twice the iron present in the entire circulating blood volume of rats of this size. Labeled glycine was then injected intravenously 1, 2, 3, 4, 7, or 11 days following the last bleeding, and the early and late peaks studied as outlined above.

Erythropoietin (sheep erythropoietin, Step I, 0.5 units/mg, Connaught Medical Research Laboratory, Toronto, Canada) was injected as a single dose of either 1 or 12 units dissolved in normal saline, via the intravenous route, to normal female LAF_1 mice. Labeled glycine was injected 50 hr after the injection of the crude erythropoietin solution.

Hypoxia was achieved in LAF_1 mice in an altitude chamber at an atmospheric pressure corresponding to an altitude of 18,000-19,000 ft above sea level. The air in the chamber was monitored for O_2 and CO_2 concentrations, which were found to be essentially the same as ordinary room air. The mice were pre-treated with injections of iron dextran to prevent emergence of iron-deficient RBC as a result of the increased erythropoietic rate, each mouse receiving 2 mg of elemental iron by subcutaneous injection 24 hr before being placed in the chamber. This is approximately 4 times the iron content of the circulating RBC of mice of this size. The mice were placed in the altitude chamber for 72 hr, following which time they were returned to

normal atmospheric pressures and injected with 10 μCi of labeled glycine each, and then returned to the altitude chamber for an additional 72 hr. They were then returned to normal atmospheric pressures for the remainder of the experiment. Breath collection was begun 4 days after the injection of the labeled glycine, so that the early peak could be studied only by method 2 in these animals.

Animals used in this study were bred from specific pathogen-free breeder stock, and splenectomy of mice and rats in the animal colony of this laboratory has failed to induce anemia, reticulocytosis, or RBC inclusion bodies characteristic of bartonella-infected animals. Although it is difficult or impossible to rule out the presence of these organisms in our animals, it appears that the animals used in these experiments do not have significant hemolysis resulting from infection with this organism.

C. Results

1) Hypoxia in the LAF_1 Mouse

Hematologic parameters for the hypoxic mice are shown in Table 1, and results of the determination of parameters of RBC survival are shown in Table 2. Although there was an unequivocal increase in hematocrit, hemoglobin concentration, reticulocyte count, and fractional incorporation of labeled glycine into RBC heme, it is apparent that there was no significant alteration of RBC survival in these hypoxic mice. Although the period of hypoxia following the injection of glycine did not allow for a complete early peak determination, the downslope of the early peak could be determined, and early peak fractionation performed by method 2 (Table 3). This component averaged 19% of total ^{14}CO production in the hypoxic mice, while the comparable figure for normal mice is 17%. Inspection of the ^{14}CO production curves in the 2 groups of hypoxic animals failed to disclose any evidence of a significant component of extremely short-lived RBC, thus ruling out the possibility of a double population of RBC or of a significant degree of ineffective erythropoiesis in these animals.

Table 1. Hemoglobin concentration, hematocrit, and reticulocyte percentage in the study animals. For the hypoxic mice, HCT and HGB were obtained 10 days after the start of hypoxia, while reticulocyte concentration was studied 3 days after start of hypoxia. For the mice treated with erythropoietin, HGB, HCT, and reticulocyte percentages were determined 5 days after injection of ESF. Values for the bled and phenylhydrazine-treated rats were obtained on the day of injection of labeled glycine. Normal values are ± 1 S.D.

Group / Time of injection of labeled glycine	Reticulocytes (%)	Hematocrit (%)	Hemoglobin (g %)
Normal LAF_1 mouse	1.6	51.6 ± 2.0	16.8 ± 0.8
Hypoxic LAF_1 mouse	6.5	60	19.6
1 unit ESF, LAF_1 mouse	5.9	53	16.9
12 units ESF, LAF_1 mouse	10.1	53	17.2
Normal buffalo rat	1.7	46.7 ± 2.0	
2 (Days after last PHH injection)	45	25	
3	80	32	
4	83	37	
5[a]	41	41	
7	64	43	
11	20	48	
Normal buffalo rat	1.7	46.7 ± 2.0	
1 (Day(s) after last phlebotomy)	22	23	
2	46	28	
3	49	31	
4	46	37	
7	16	47	
11	9	47	

[a]This animal was studied and injected at a different time from all the other phenylhydrazine-treated rats.

Table 2. Parameters of RBC survival in the study animals. Normal values are ± 1 S. E.

Group / Time of injection of labeled glycine	Mean potential life span (MPLS) (days)	Standard deviation of life spans about MPLS (days)	Random hemolysis (%/day)	Incorporation of glycine into circulating RBC heme (%)
Normal LAF_1 mouse	51.8 ± 0.4	9.1 ± 1.2	0.60 ± 0.17	0.32 ± 0.01
Hypoxic LAF_1 mouse, group I	52.8	8.5	0.61	0.99
Hypoxic LAF_1 mouse, group II	52.6	8.5	0.68	0.93
1 unit ESF, LAF_1 mouse	49.5	12.1	0.28	0.63
12 units ESF, LAF_1 mouse, group I	49.5	8.4	0.39	0.60
12 units ESF, LAF_1 mouse, group II	50.3	7.8	0.64	0.82
Normal buffalo rat	66.2 ± 0.7	7.6 ± 0.6	0.67 ± 0.07	0.25 ± 0.03
2 (Days after last PHH injection)	43.9	9.5	1.03	1.96
3	51.0	8.1	0.79	2.45
4	54.9	6.6	0.45	2.22
5[a]	58.1	7.5	0.54	1.31
7	56.9	7.1	0.38	1.44
11	62.6	9.4	0.35	0.78
Normal buffalo rat	66.2 ± 0.7	7.6 ± 0.6	0.67 ± 0.07	0.25 ± 0.03
1 (Day(s) after last phlebotomy)	52.9	6.1	0.64	1.23
2	48.9	8.1	0.44	1.77
3	55.9	8.6	0.41	1.63
4	56.6	7.4	0.39	1.54
7	60.2	9.3	0.46	1.27
11	65.9	7.6	0.45	0.53

[a] This rat was studied and injected with phenylhydrazine at a different time from all the other rats in the group.

Table 3. Early peak fractionation in study animals. Values given are for the ratio of the early peak to total ^{14}CO production, as calculated either by method 1 or method 2 (see Materials and Methods). Normal values are ± 1 S. E.

Group / Time of injection of labeled glycine	Ratio: early peak to total ^{14}CO production	
	Method 1	Method 2
Normal LAF_1 mouse	0.18-0.31	0.17
Hypoxic LAF_1 mouse	--	0.19
1 unit ESF, LAF_1 mouse	0.24	--
12 units ESF, LAF_1 mouse	0.25	--
Normal buffalo rat	0.20-0.30	0.29±0.09
2 Days after last PHH injection	--	0.38
3 Days after last PHH injection	0.20	0.31
4 Days after last PHH injection	--	0.17
5 Days after last PHH injection	0.13	0.13
7 Days after last PHH injection	--	0.17
11 Days after last PHH injection	--	0.16
Normal buffalo rat	0.20-0.30	0.29±0.09
1 Day(s) after last phlebotomy	--	0.11
2 Day(s) after last phlebotomy	--	0.07
3 Day(s) after last phlebotomy	0.12	0.05
4 Day(s) after last phlebotomy	--	0.06
7 Day(s) after last phlebotomy	--	0.09
11 Day(s) after last phlebotomy	--	0.11

2) Erythropoietin Stimulation in the LAF_1 Mouse

Results for these experiments are shown in Tables 1-3. They indicate that although a single injection of 1 or 12 units of crude sheep ESF did not significantly alter hemoglobin concentration or hematocrit 5 days after injection, a significant increase in reticulocyte percentage and increased fractional glycine incorporation was achieved. Parameters of RBC survival (Table 2) were somewhat irregularly changed in these animals, but none were significantly different. Examination of total ^{14}CO production by method 1 showed a normal early peak fractionation in all experimental groups, without evidence for increased ineffective erythropoiesis or the production of a component of extremely short-lived RBC.

3) Phenylhydrazine-Induced Anemia in the Rat

Results for these experiments are shown in Tables 1-3 and Fig. 1 and document a maximal anemia on day 4, maximal glycine incorporation on day 5, and a maximal reticulocytosis on day 6, following injection of this drug on days 0, 1, and 2. Red blood cell survival patterns show that the only statistically significant changes have occurred in the mean potential life span, with a maximum shortening to 65% of normal in the cohort of cells labeled 2 days after the last injection of phenylhydrazine. Thereafter, the mean potential life span returned towards normal values at approximately the same rate as the venous hematocrit returned towards normal. Eleven days after the last injection of phenylhydrazine, when the hematocrit had returned to normal, the reticulocyte percentage was still markedly elevated, fractional glycine incorporation was more than 3 times normal, while the mean potential life span was only 4% shorter than normal.

Total ^{14}CO fractionation, as measured by either method 1 or 2, showed that the ratio of the early peak to total ^{14}CO production was increased in the cohorts labeled 2 days after the last injection of PHH, normal in the cohort formed 3 days after the last dose, and subnormal in subsequent cohorts, although none of the changes are statistically significant because of the great variability of this ratio in the normal rats. Detailed examination of the shape of the early peak disclosed

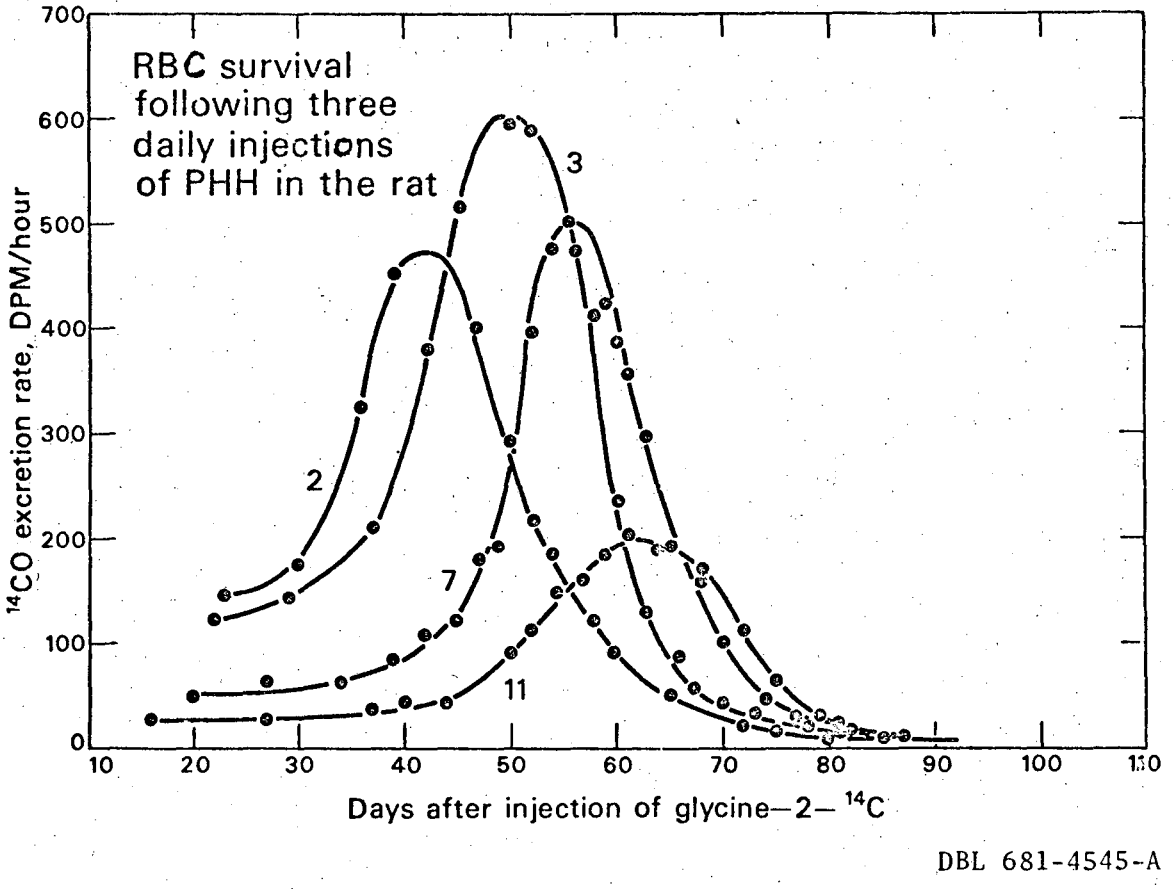

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Fig. 1. Serial changes in ^{14}CO production during recovery from phenylhydrazine (PHH)-induced anemia. Graph shows ^{14}CO production in individual rats (dpm/hr, ordinate) vs. time after injection of glycine-2-^{14}C (days, abscissa). Numbers beside each curve indicate the interval of time (days) between the last of three consecutive daily injections of PHH and the time of glycine injection. Note the progressive return towards normal of the peak of ^{14}CO production during recovery.

an indistinct but constant alteration during the first 24 hr following injection of labeled glycine only in the animal injected with glycine 3 days after the last injection of PHH. The changes, which were not present in the study conducted 5 days after the last dose of PHH, consisted of a displacement of the time of maximum excretion of ^{14}CO from the normal 2-3 hr to about 6 hr (Fig. 2), with an indistinct secondary rise 9-14 hr after glycine injection. Other than these early peak alterations, there was no evidence for a significant population of RBC with an extremely short life span.

Results of the cross-transfusion experiments are shown in Table 4. Normal, labeled RBC survive normally in a normal rat host, with the exception of a shortening of the mean potential life span of about 6%. However, cells produced in a phenylhydrazine-treated rat had a markedly altered survival pattern when transfused into a normal host. Mean potential life span shortening of about 10% was seen, but there was also the presence of a marked increase in random hemolysis, the maximum observed rate being 60 times normal. In addition, hemolysis of the washed, labeled cells was noted in vitro, whereas this was not seen with the normal cells. In all, there were up to three components of random hemolysis in the cross-transfused phenylhydrazine-treated donor cells, instead of the usual one. Most of these cells were destroyed in the first 2 weeks following the cross-transfusion, but the cells remaining in the circulation were still subjected to increased rates of random destruction up to twice normal.

4) Blood Loss Anemia in the Rat

Previous studies (15) have shown that pretreatment of bled rats with iron dextran prevents the production of low serum iron levels in these animals. In one of the animals studied in this series, the serum iron concentration on the day following the last of three daily phlebotomies, in a rat pretreated with iron dextran, was 267 micrograms percent (normal control: 168 micrograms percent).

Results of this study show a maximal anemia on day 3, maximal fractional glycine incorporation on day 4, and maximal reticulocytosis on day 5, when phlebotomy was performed on days 0, 1, and 2. Again,

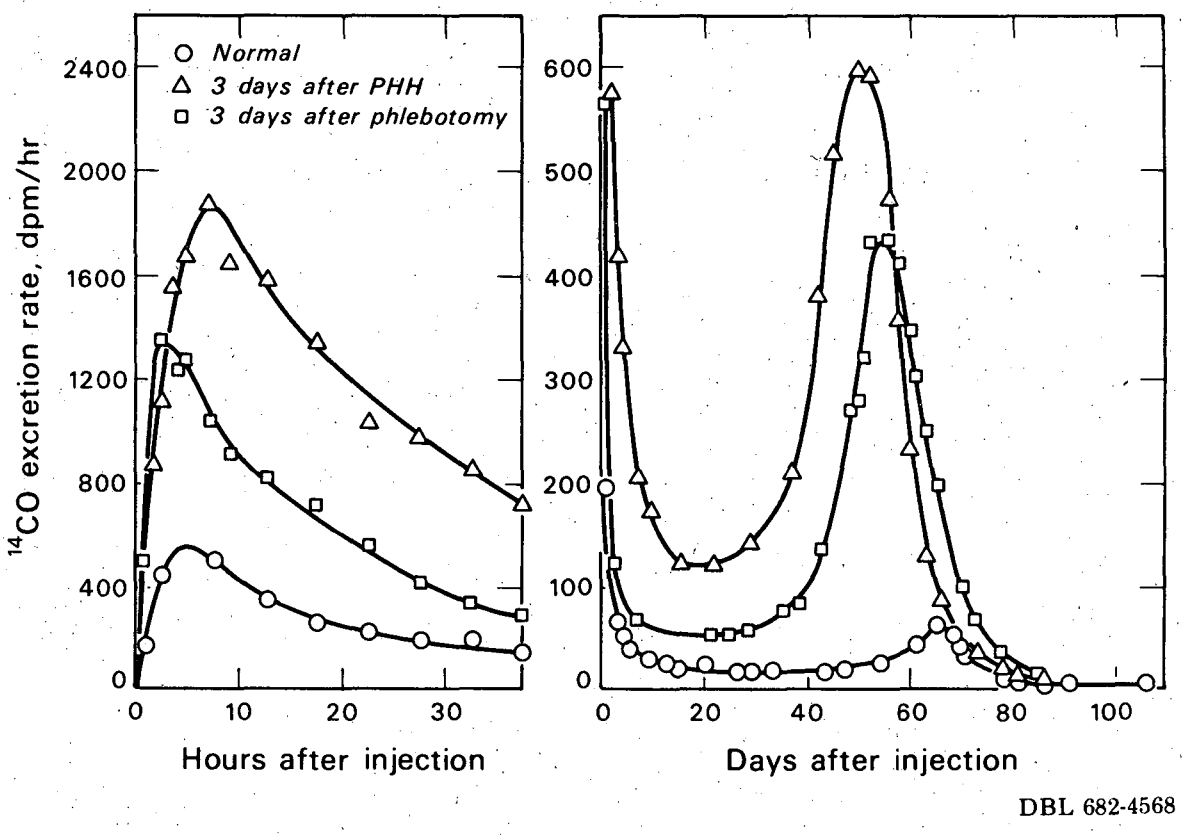

Fig. 2. ^{14}CO production in a normal rat, a phlebotomized rat, and a phenylhydrazine-treated rat. Note the difference in the ordinate and abscissa scales for the time periods 0-35 hr and 1-100 days following injection of labeled glycine. Labeled CO excretion rates are expressed in dpm of ^{14}CO per hour.

Table 4. Parameters of RBC survival in cross-transfusion experiments. Normal values given are ± 1 S. E.

Group	Mean potential life span (MPLS) (days)	Standard deviation about MPLS (days)	Random hemolysis (%/day)
Normal control animals	66.2 ± 0.7	7.6 ± 0.6	0.67 ± 0.07
Normal donor A cross-transfused into normal host I	63.2	9.4	0.58
Normal donor B cross-transfused into normal host II	62.1	8.3	0.44
Glycine injected 3 days after last of three consecutive daily doses of phenylhydrazine:			
Survival in original host	51.0	8.1	0.79
Survival of donor C cells after cross-transfusion into normal host III	46.3	6.0	1.35, 6.5, 35.0
Survival of donor C cells after cross-transfusion into normal host IV	46.6	6.1	0.97, 12.0[a]

[a]This animal was not studied until the 8th day after cross-transfusion, so that an earlier, third component of random hemolysis, if present, could not be detected.

parameters of RBC survival were unchanged with the exception of statistically significant shortening of the mean potential life span, which was maximally shortened to 75% of normal in the cohort produced 2 days following the last phlebotomy (day 4) (Fig. 3, Table 2). Thereafter the mean potential life span returned towards normal at a rate approximating the rate of return of the venous hematocrit towards normal. Eleven days after the last phlebotomy, at a time when the venous hematocrit had been normal for at least the preceding 4 days, the reticulocyte percentage was still elevated and the fractional glycine incorporation was twice normal, although RBC survival was entirely normal.

Fractionation of total ^{14}CO production by methods 1 and 2 showed that the early peak-total CO ratio was below normal in all the bled animals. There was no evidence of an alteration in the shape of the early peak (Fig. 2), and no evidence for a population of extremely short-lived RBC in any of the rats so treated.

D. Discussion

The method used in this study for evaluating RBC survival in animals with stimulated erythropoiesis offers distinct advantages over techniques previously used. Subsequent to most forms of erythropoietic stimulation there is a significant change in total body RBC volume. Thus, when RBC survival is studied by measurement of circulating heme specific activity, independent measures must be made of total circulating RBC mass in order to correct for changes due to an expanding or contracting RBC mass. The ^{14}CO method yields results which are independent of these changes, since it measures only the catabolism of heme in the cohort of cells labeled following the injection of glycine-2-^{14}C. The necessity of repeated anesthesia and venesections, which may in themselves alter erythropoiesis, is avoided with the method used in this study. The cross-transfusion studies have shown *in vitro* and *in vivo* hemolysis of the cells formed shortly after the cessation of phenylhydrazine treatment. Therefore, it is possible that techniques requiring blood sampling, handling, and washing of cells prior to deter-

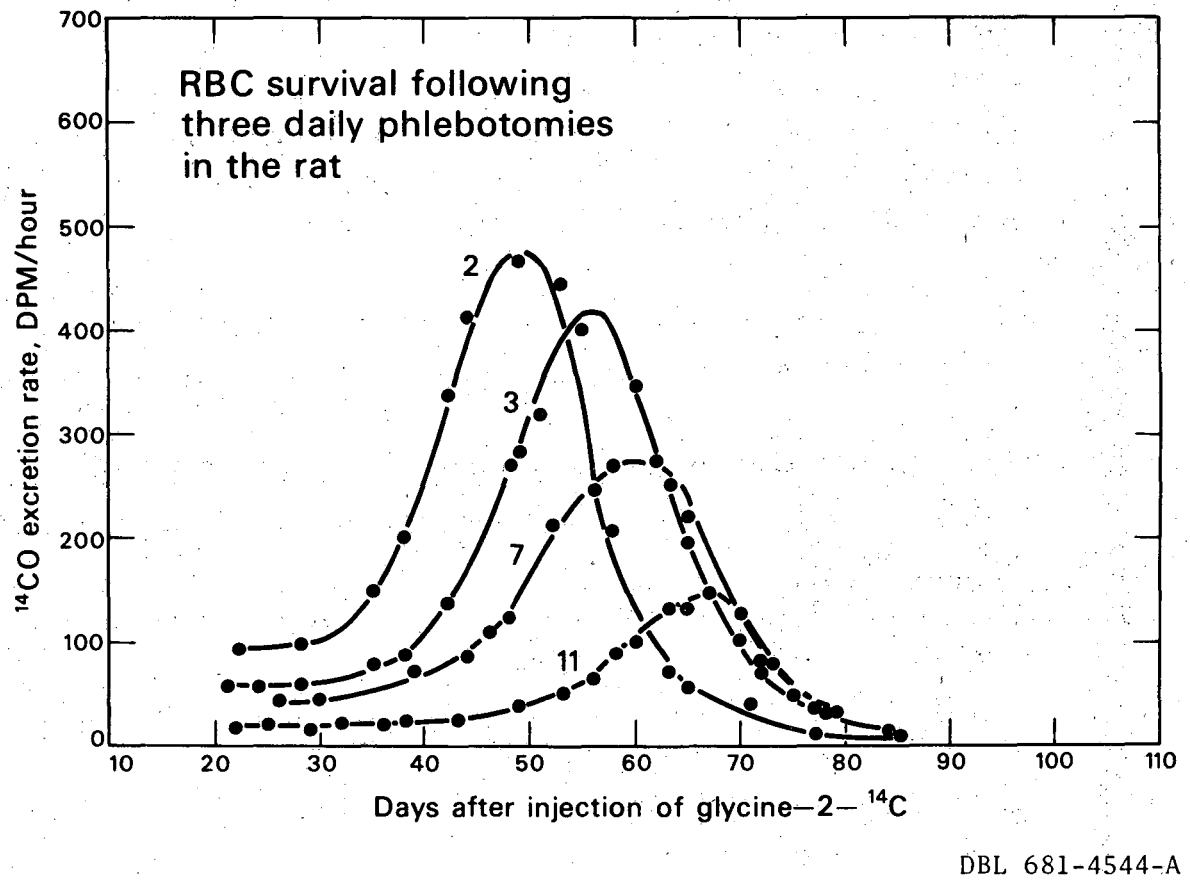

Fig. 3. Serial changes in ^{14}CO production during recovery from anemia secondary to repeated phlebotomies. Ordinate and abscissa same as for Fig. 1. Numbers beside each curve indicate the interval of time (days) between the last of three consecutive daily phlebotomies and the time of glycine injection. A progressive return towards normal of the peak of ^{14}CO production is again seen during recovery.

mining heme specific activity may actually be destroying some of the more fragile labeled cells before they can be counted.

While the ^{14}CO method provides a means for estimating the fraction of labeled heme degradation associated with ineffective hemoglobin synthesis and non-hemoglobin heme turnover (early peak), as well as determination of the parameters of survival of circulating RBC, there are a number of inherent approximations and assumptions. Quantitative fractionation of the early peak into its two main components is not yet possible. Since the turnover of hemoglobin heme in the marrow and non-hemoglobin hemes in the rest of the body may be totally independent processes, and since it is only the ratio of the former process to the "late peak" that is of interest in this regard, a better understanding of ineffective erythropoiesis awaits the improved definition of this fractionation.

When the size and gross turnover rates of the glycine pool are constant, the relative fraction of labeled glycine incorporated into circulating RBC heme is a good measure of the relative increase in effective hemoglobin synthesis. In the present experiments, some of the erythropoietic stimuli might be associated with an overall increase in the net turnover rate of the glycine pool. Under these circumstances, the relative fractional glycine incorporation underestimates the increase in hemoglobin synthesis. One can thus conclude that the relative increase in effective hemoglobin synthesis is either equal to or greater than the relative fractional glycine incorporation into circulating RBC heme.

Exposure of female LAF_1 mice to 6 days at a simulated altitude of 18,000 ft, or injection of a single dose of 1 or 12 units of crude sheep erythropoietin increased RBC production, as evidenced by increases in reticulocyte count and fractional glycine incorporation, with the maximum glycine incorporation being slightly less than 3 times normal. In the hypoxic mice, parameters of RBC survival were entirely normal. There was a minimal decrease in the mean potential life span in the mice treated with the crude sheep erythropoietin, with greater shortening found at the higher dosage level. There was no consistent evidence of increased ineffective erythropoiesis, or early RBC death in the mouse experiments.

Three daily injections of phenylhydrazine (PHH) or three daily phlebotomies in the buffalo rat produced almost identical changes in the venous hematocrit, but marked differences in reticulocyte percentage and fractional glycine incorporation. Examination of the results shown in Tables 1 and 2 reveals that rats treated with either PHH or phlebotomy, with either comparable hematocrits or reticulocyte percentage, do not show comparable alterations in the mean potential life span, the only parameter showing statistically significant alterations. It is only when the fractional incorporation of glycine is comparable that the mean potential life span shortening is also comparable. Figure 4 shows that there is nearly a linear increase in the degree of shortening of the mean potential life span with increased fractional glycine incorporation in the range from 3-10 times normal. The same figure shows that increases in glycine incorporation of less than 3 times normal are associated with minimal or no changes in mean potential life span, results that are in agreement with some of the previously published studies (7, 8, 9). The points on the graph which show the linear relation least well are from animals in which the fractional glycine incorporation was still submaximal (1 and 2 days after the last of 3 phlebotomies, and 2 days after last injection of PHH), suggesting that the earliest cells produced following these stresses are more abnormal than those cells produced subsequently. This finding is in general agreement with recent theories of response to such stimuli, which state that the initial response to intense erythroid stimuli may be achieved without an increase in marrow cellular components (17), by decreasing intramedullary maturation time and skipping terminal divisions (18, 19), while maximal responses are obtained by increasing total marrow cellularity, which allows for increased RBC production without producing the markedly abnormal cells seen initially.

Examination of the shape of the early peak in all the experimental animals revealed no significant alterations from normal with the exception of the animals studied less than 4 days after the last of 3 daily injections of phenylhydrazine. The qualitative and quantitative changes in the latter instance suggest a moderate increase in ineffective eryth-

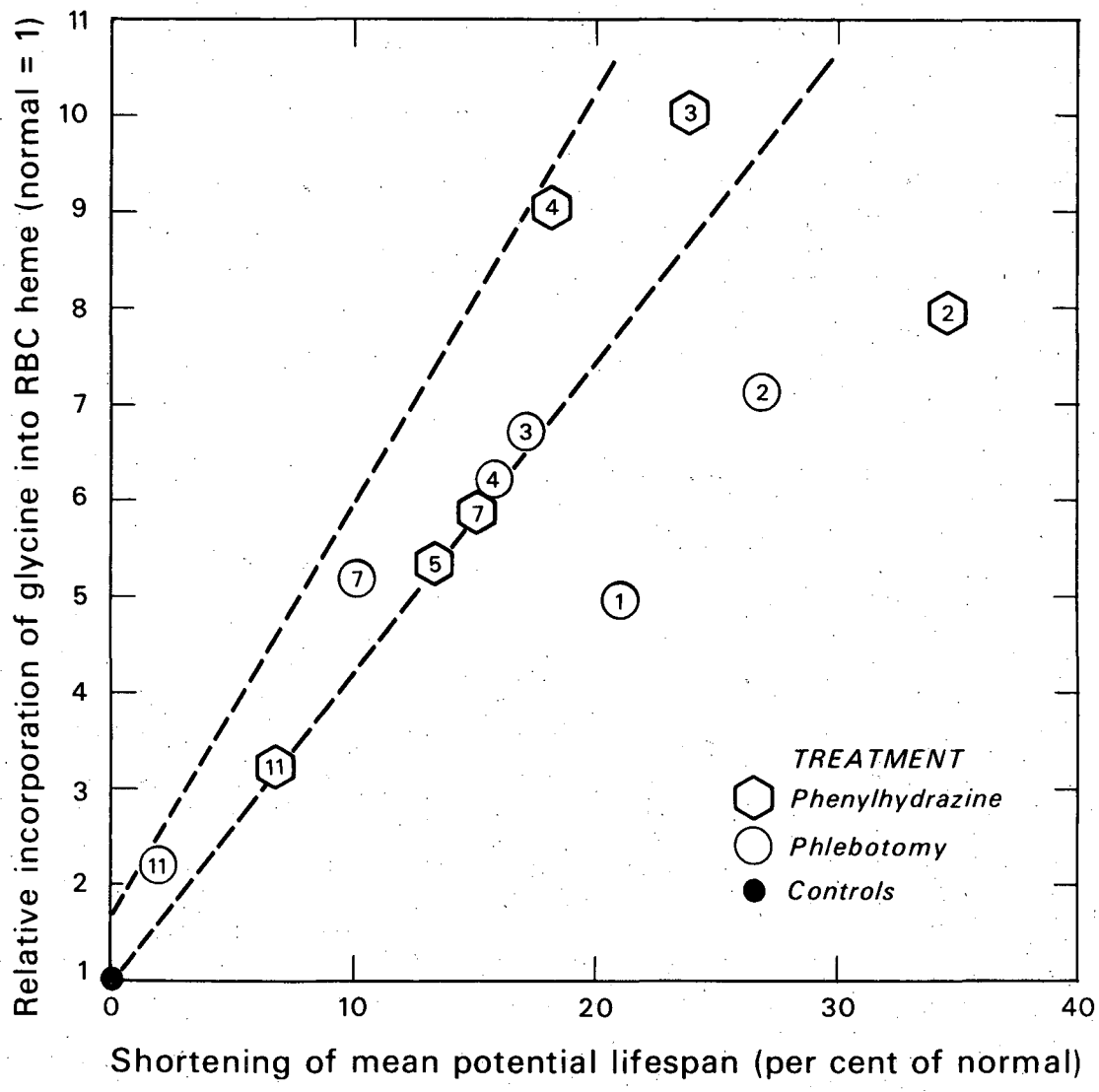

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Fig. 4. Graph showing the relationship between the shortening of the mean potential life span (as percent of normal, abscissa) and the relative incorporation of glycine-2-^{14}C into circulating RBC heme (ordinate) for phlebotomized (circles) and phenylhydrazine-treated (hexagons) rats. Numbers inside symbols indicate the length of time (days) between the last injection of phenylhydrazine or the last phlebotomy and the injection of labeled glycine. Note that those experimental points falling outside of the area enclosed by the dotted lines all involve intervals of time of 2 days or less.

ropoiesis, which occurred along with fractional glycine incorporation approximately 6 times normal. Since these changes were not seen in a bled animal with a similar fractional glycine incorporation, this is most probably due to a direct toxic effect of the drug on developing erythroid cells. In all other instances, early peak:total CO fractionation was either normal or subnormal, suggesting that erythropoiesis becomes more effective as erythropoietic rate increases above normal. However, as noted earlier, until the early peak can be accurately described in terms of its two general components, this fractionation will have only grossly qualitative significance in the study of ineffective erythropoiesis.

The cross-transfusion experiments document that the changes herein described are due to an intrinsic defect in the RBC (Table 4), but also point out the fact that the cells produced in a PHH-treated rat do not survive normally following the cross-transfusion procedure, more than one-third of the transfused cells being destroyed by random processes before the third week after labeling. Similar results have recently been presented by Robinson (20), who found that of normal cross-transfused labeled RBC, 1.6% of the labeled heme was converted into bile pigment in 3 days, while in cross-transfused labeled cells produced following phlebotomy, 7.6% was converted to bile pigment in the same time period. These results are fully compatible with the observation that newly formed RBC are markedly fragile in vitro, although the in vivo studies did not show increased rate of random destruction, suggesting an artifactual nature of the observations in the cross-transfused animals.

The maintenance of a finite, but minimally shortened (less than 35%) mean potential life span, without concomitant evidence of ineffective erythropoiesis (except as noted in one animal), production of a double population of cells, or increased random hemolysis speaks for functional normalcy of these cells. That they maintain their normally distributed survival about a shorter mean life span suggests that they have simply aged faster than normal. Whether this increased aging is due to a decreased amount of a critical material initially given to the

cell, or to an increased rate of degradation of a critical component is not known.

Recent work (21) has shown that non-hemoglobin protein formation in the fetal mouse erythron is directly under the control of messenger RNA, since it can be almost completely suppressed with actinomysin. However, actinomysin does not depress hemoglobin formation in the same cells. Thus, when the nucleus is extruded from the cell, it is possible that non-hemoglobin protein synthesis stops abruptly, while hemoglobin synthesis continues. Since the enzymes of the pentose-phosphate shunt and Embden-Myerhof pathways are included in this former group, and are of critical importance in maintaining normal survival (22), any shortening of intramedullary time will most likely decrease the time over which these enzymes can be manufactured, leading to decreased levels in the emerging RBC. Under these circumstances, one might expect the emerging cell to reach a critically low content of these materials sooner than normal, resulting in a decreased mean potential life span.

E. Summary

Red blood cell survival was studied in LAF_1 mice and buffalo rats following hypoxia, erythropoietin injection, repeated phlebotomy, or phenylhydrazine-induced anemia, using a technique based upon the endogenous production of ^{14}CO in the breath following the injection of glycine-2-^{14}C. Despite estimated hemoglobin synthesis rates 10 or more times normal, in no instances were there any increases in the rate of random destruction of the produced RBC, nor was there the production of a component of extremely short-lived cells. Ineffective hemoglobin synthesis appeared to be either normal or decreased, with the exception of rats studied shortly after injection of phenylhydrazine, suggesting a toxic effect of the drug in this time period. A modest but significant shortening of the mean potential life span (senescent destruction) was seen, which increased linearly with increasing hemoglobin synthesis rate. This shortening never exceeded 35% and no shortening of any significant degree was seen unless estimated hemoglobin synthesis

rates were more than 2-3 times normal. The minimal alterations in RBC survival noted in these experiments despite marked erythropoietic stimulation lead one to conclude that these cells are remarkably normal.

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