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Endogenous Production of Carbon-14 Labeled Carbon Monoxide: An *In Vivo* Technique for the Study of Heme Catabolism[1]

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INTRODUCTION

In the late 1940's, Sjöstrand demonstrated the presence of carbon monoxide in the expired air of the normal, non-smoking 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 this carbon monoxide arose directly from the alpha-methene bridge carbon of heme.

It has been established that the metabolic precursor of the four methene bridge carbon atoms of heme is the methylene carbon (carbon-2) of glycine (4), and several investigators have been able to recover carbon-14 labelled 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.

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METHODS AND INSTRUMENTATION

A. *Standardization of Radioactive Gases:*

Standard tanks of ^{14}CO and $^{14}CO_2$ were obtained by adding the labelled gases (New England Nuclear, Boston, Massachusetts) 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 $^{14}CO_2$ gases were passed through a 22-liter ionization chamber operating at atmospheric pressure, with a collecting potential of 300 volts. The signal from this chamber was passed to a vibrating reed electrometer (Model 30, Applied Physics Corporation, Pasadena, California) 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/millivolt for a 10^{12} ohm resistor. A signal of 1 millivolt 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 IA.

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 ($\pm$ 2%), error of reading the potentiometer signal, changes in ambient temperature and barometric pressure, plus any deviation from ideality in the counting efficiency. No attempt was made to correct for the small variations in temperature and pressure noted during this period. 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 IA are probably accurate only to within 5-10%.

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 counting by liquid scintillation. 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 ml aliquots of this absorber solution were added to 15 ml of a scintillator solution made of a 1:2 (v/v) solution of ethylene glycol monomethyl ether in toluene, containing 5.50 grams per liter of 2,5-diphenyloxazole (PPO, Scintillation Grade, Packard Instrument Company, Downers Grove, Illinois). The resulting homogeneous, 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, Massachusetts). Typical counting efficiencies (cpm/dpm) were in the range of 0.497–0.510. Background ranged from 34 to 43 cpm. Table IB shows the results obtained using this method. Standard ^{14}CO gas was found to be completely *insoluble* in the absorber 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 the internal standard (2.4% for 99.5% confidence), and flowmeter error (0.5%). Temperature and pressure variations were again neglected. The values calculated in Table IB are probably accurate to within 3%.

The value obtained for the $^{14}CO_2$ standard gas 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 Figure 1.

TABLE I

CALIBRATION OF THE ^{14}CO AND $^{14}CO_2$ STANDARD GASES

TABLE IA

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 IB

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)

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

The metabolism cage is made of a clear plastic, with a volume of approximately 2 liters. It can hold a 350-450 gram 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 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 chamber system as previously described under Part A., Method I. Air is continuously evacuated from the chamber by means of a constant-flow diaphragm pump (N. V. Godart, Netherlands). The air is then passed through a sodalime cannister containing approximately 250 grams of sodalime, and 100 grams 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.

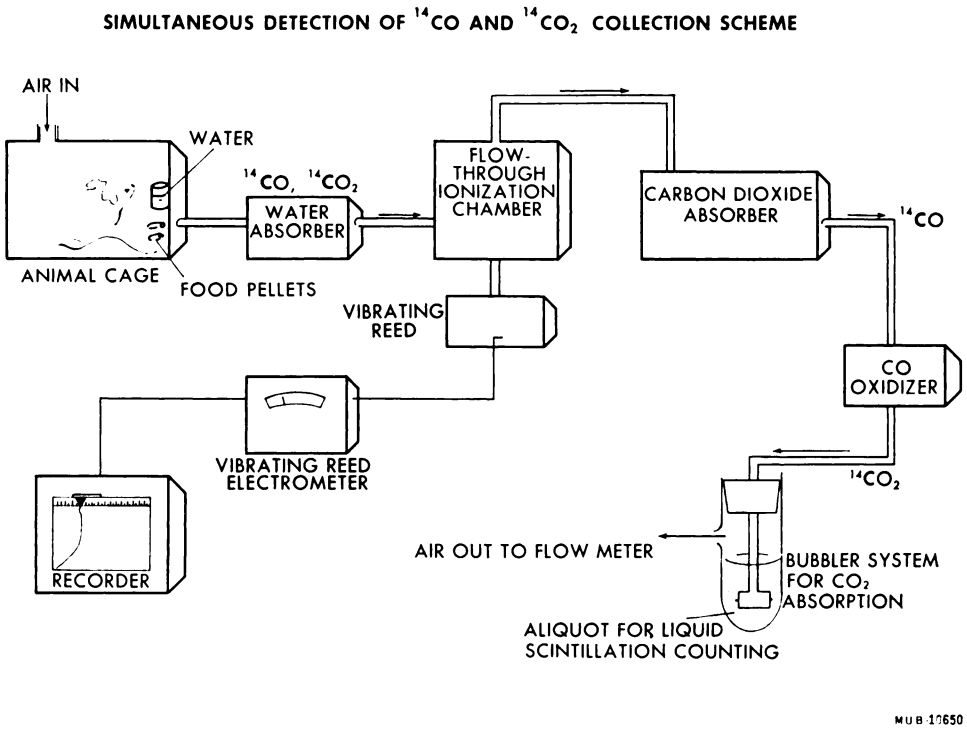

Fig. 1. In Vivo Breath Collection System. Schematic representation of apparatus used to measure ^{14}CO and $^{14}CO_2$ production simultaneously and continuously in the intact rat. The radioactive gases present in the air stream are noted for three different portions of the apparatus. The separate components of the system are described in the text. Not shown is the constant-flow diaphragm pump, which is placed between the ionization chamber and the carbon dioxide absorber.

The air then passes through a cannister containing approximately 17 grams of Hopcalite (Mine Safety Appliances Company, Pittsburgh, Pennsylvania, 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 absorber solution previously described. The air existing from the absorption tube is passed through a wet-test meter (American Meter Company, Albany, New York) for determination of flow rates.

Animals used for experimentation were male buffalo rats, weighing 340 to 370 grams, corresponding to an age of approximately 3-4 months, Glycine-2-^{14}C was purchased in vials containing 50 μCi/ml. in sterile saline, with a specific activity of approximately 22 mC/mM (New England Nuclear, Boston, Massachusetts). On chromatographic analysis all of the material was contained in one homogeneous peak characteristic of glycine. Contamination by chromatographically similar materials was not excluded. Fifty microcuries were injected I.V. into each animal under light ether anesthesia. The animal was placed inside the metabolism cage immediately, and breath collection started within 1 minute of the time of injection. The animals were fully reactive, and radioactivity of the expired air was noted 3-5 minutes 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 per minute. At these flow rates, the animals were apparently comfortable, and there was moderately vigorous bubbling in the gas absorber tube.

TECHNIQUE ANALYSIS

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

For the first experiment, 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 an absorption capacity of the absorber solution for CO_2 on the order of 1.8 millimoles per ml, 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 one week, if all the carbon monoxide is oxidized to carbon dioxide by the Hopcalite. Ten ml 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/hour at a flow of 0.4 liters per minute. 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 due to evaporation was measured, it amounted to less than 0.3%, and was thus neglected.

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 minutes, and, when necessary, for longer periods of time to insure a maximum of 1% 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/hour or μCi/hour.

II. *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 Figure 1. Results are shown in Table II. It is apparent that oxidation efficiency approaches 100% at the highest CO concentration (5000 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 II 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.

III. *Carbon Dioxide Absorption Efficiency of Sodalime Absorber*

One of the important requirements of the system depicted in Figure 1 is that the radioactive CO_2 must be quantitatively trapped in the sodalime, 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 sodalime 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 sodalime, 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 sodalime 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.

IV. *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 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 sodalime 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 sodalime 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 due to $^{14}CO_2$ is so small.

3. At the times noted on Figure 2, the sodalime 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 included in the graphic display for this material 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 sodalime and Drierite cannisters.

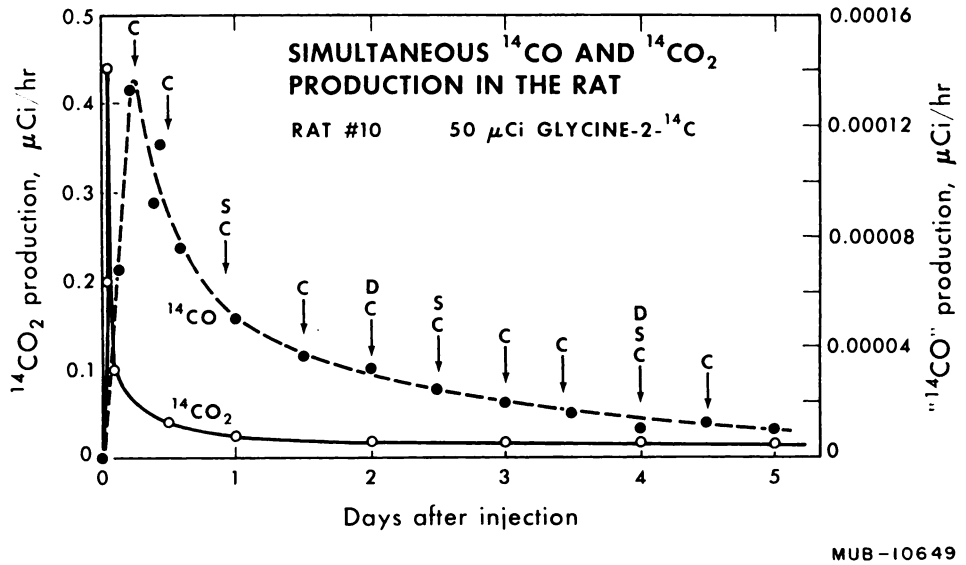

Fig. 2. Simultaneous $^{14}Co_2$ and "^{14}CO" Production in the Rat. The graph shows the rate of appearance of $^{14}CO_2$ (open circles, left ordinate) and "^{14}CO" (closed circles, right ordinate) in the expired air of a normal buffalo rat following I.V. injection of 50 μCi of glycine-2-^{14}C, as obtained by the apparatus shown in Figure 1. The factor of 3000 difference in the ordinate scales should be noted. Arrows refer to times when the various components of the system were changed, as follows: C = Metabolism Cage, S = Sodalime Cannister (CO_2 absorber), D = Drierite Cannister (water absorbed).

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 hours 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 Figure 1. Air was passed over this material for 5 hours 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 were used in place of the urine and feces, while in the third experiment, 2 μCi of acetone-2-^{14}C were 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 labelled acetone produced by a *non-fasted* 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 red blood cell 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 injection of the labelled glycine.

DISCUSSION

The functioning of this system depends upon four important assumptions. They are:

1. Respiratory $^{14}CO_2$ can be completely absorbed in the sodalime 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 labelled 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 sodalime absorber allows passage of

about 1 part in 75,000 of the $^{14}CO_2$. Thus, the efficiency of the sodalime 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 labelled with Carbon-14 after injection of glycine-2-^{14}C. Experiments showed that only 1 part in 75,000 of the $^{14}CO_2$ escapes the sodalime absorber. Two of the ketone bodies, acetoacetic acid and beta hydroxy butyric acid, being acids, are likely to be absorbed by the sodalime 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 labelled 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%, white 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 labelled 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 red blood cell life span of the rat, using other techniques.

(c)—The amount of activity absorbed in ethanolamine during the first five days after injection of labelled 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 labelled" 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 labelled" bilirubin in dogs with normal, increased, and decreased erythropoiesis (19).

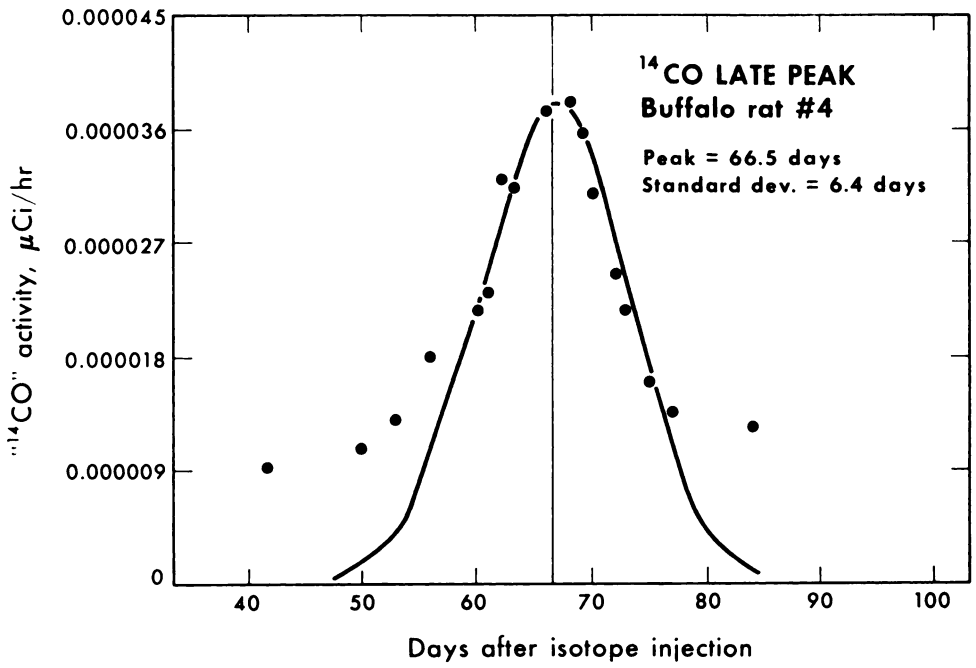

Fig. 3. "Late" Production of "^{14}CO" in a Normal Buffalo Rat. The rate of appearance of "^{14}CO" in the expired air 40 to 90 days after administration of 50 μCi of glycine-2-^{14}C to a normal buffalo rat is shown. Experimental points are indicated by closed circles. A "normal" or Gaussian curve is 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 red blood cell life spans about the mean value for a normal buffalo rat.

TABLE II

HOPCALITE OXIDATION EFFICIENCY

Experiment Number	*CO Concentration (PPM)*	*Calculated Oxidation Efficiency (%)* **
1	5000	97.8
2	5000	97.8
3	5000	96.4
*4	25	88.7
*5	5	83.9

*See text for discussion of errors

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

3. The oxidation efficiency of Hopcalite was shown to be greater than 80% over the range of concentrations tested (Table II). 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-hour collections, the longest intervals routinely used.

The dose of 50 μCi was chosen so that the "^{14}CO" 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 (22mC/mM) 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 per hour in a 350 gram rat (21), this dose is probably still in the "tracer" range.

SUMMARY

A method is presented for the separation, detection, and quantitation of endogenously produced carbon-14 labelled 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 sodalime 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 should provide a unique source of information in the study of disease processes characterized by abnormal heme catabolism in man and other animals.

ADDENDUM

More accurate results are now available on the oxidation efficiency of Hopcalite for ^{14}CO than those presented in Table II for gases with CO concentrations of 25 and 5 ppm. Using an *undiluted* standard ^{14}CO gas with a CO concentration of less than 2 ppm, it was found that the oxidation efficiency was 99.6% at a flow rate of 0.30 l/min, and 98.0% at a flow rate of 0.37 l/min. Thus, the high efficiency of oxidation shown in Table II for gases with a high concentration of CO (5000 ppm), is also found for gases with CO concentrations approximating that found in the expired air of mammals.

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