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Carbon Monoxide Production Associated with Ineffective Erythropoiesis *

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Abstract. The rate of endogenous carbon monoxide production ($\dot{V}_{CO}$), determined by the closed rebreathing system technique, was elevated above the normal range in four of five patients studied with ineffective erythropoiesis (four patients with primary refractory anemia, one with thalassemia). The mean molar ratio of $\dot{V}_{CO}$ to $\dot{V}_{heme}$ (rate of circulating heme catabolism, determined from ^{51}Cr red cell survival curves) was 3.0 ± 0.6 (SE), indicating that most of the CO originated from sources other than circulating erythrocyte hemoglobin, in contrast to previous findings in patients with hemolytic anemia, where $\dot{V}_{CO}$ paralleled $\dot{V}_{heme}$ closely.

After administration of glycine-2-^{14}C to these patients, endogenous CO was isolated by washout of body CO stores at high pO_2 or by reacting peripheral venous blood samples with ferricyanide. The CO was then oxidized to CO_2 by palladium chloride and trapped for counting in a liquid scintillation spectrometer. "Early labeled" peaks of ^{14}CO were demonstrated which paralleled "early labeled" peaks of stercobilin and preceded maximal labeling of circulating heme. Production of "early labeled" ^{14}CO in patients with ineffective erythropoiesis was greatly increased, up to 14 times that found in a normal subject.

The increased $\dot{V}_{CO}$ and "early ^{14}CO" production shown by these patients are presumably related mainly to heme catabolism in the marrow. The possibility exists that hepatic heme and porphyrin compounds may also contribute significantly to $\dot{V}_{CO}$, as suggested by the finding of a high $\dot{V}_{CO}$ in an additional patient with porphyria cutanea tarda.

Introduction

Since the studies reported in 1950 by London et al. and Gray et al. (1, 2), it has been known that 10–20% of bile pigment produced in normal subjects is derived from sources other than the hemoglobin of circulating erythrocytes. This fraction of bile pigment is excreted predominantly as the so-called "early labeled" peak of stercobilin shortly

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after the administration of labeled glycine. The "early labeled" peak is increased when erythropoiesis is stimulated by phlebotomy (3) and decreased when erythropoiesis is depressed (4), suggesting that "early labeled" bile pigment arises within the marrow, either by destruction of a minor population of defective red cell precursors, or by release of a minor fraction of newly synthesized hemoglobin from within maturing red cells (5).

More recently, evidence from several laboratories has indicated that rapid turnover of heme-containing enzymes in the liver also contributes to "early labeled" bilirubin (6–9), so that the relative contribution of the marrow to the "early labeled" peak in normal man is uncertain at the present time. The "early labeled" peak is grossly exaggerated in certain diseases accompanied by abnormal erythropoiesis, however, such as pernicious anemia (10), erythropoietic porphyria (2, 11), and thalassemia (12), and some cases of primary refractory anemia (4, 13). In such patients, it seems likely that the "early labeled" peak of bile pigment arises predominantly within the marrow, by destruction of abnormal erythrocyte precursors. These patients characteristically show erythroid hyperplasia of the marrow, excrete increased total amounts of bile pigment, and have supranormal rates of plasma iron turnover. The increase in heme synthesis implied by these findings, however, is not reflected in production of circulating red cell hemoglobin, as measured by ^{59}Fe reappearance or ^{51}Cr red cell survival studies (14, 15). In this anomalous situation, termed ineffective erythropoiesis to connote wasted heme synthesis by the marrow, the important problem of quantitating total heme turnover has remained a difficult one. While the presence of an increased "early labeled" peak of stercobilin has been the hallmark of ineffective erythropoiesis, isotopic studies with ^{14}C-labeled precursors do not provide a convenient method for measuring heme turnover. Calculation of heme turnover from fecal bile pigment excretion has proved erratic, moreover, since degradation of urobilinogen to unidentifiable products either in the gut or after reabsorption leads to unpredictable deficits in recovery (16). The possibility has also been raised that alternate pathways in heme catabolism may exist which bypass the usual urobilinogen route (17), leading to an underestimation of heme turnover.

In view of these difficulties, we have thought it worthwhile to evaluate the use of endogenous carbon monoxide (CO) production as an index of heme turnover in states of ineffective erythropoiesis. CO has been shown to be a normal end product of human metabolism (18, 19) and its rate of production ($\dot{V}co$) in normal subjects is consistent with the concept that hemoglobin catabolism provides its major source. CO release during heme degradation has been demonstrated using in vitro models (20), and isotopic studies have shown that CO most probably arises from the alpha methine bridge carbon atom of the porphyrin ring in the degradation of heme in vivo (21). Studies of CO metabolism in normal subjects injected with damaged erythrocytes and in patients with hemolytic anemia have shown a close correlation between $\dot{V}co$ and the rate of circulating heme catabolism (22, 23). These findings imply that CO release during heme breakdown is stoichiometric and that recovery is nearly quantitative. Additionally, evidence from animal experiments indicates that conversion of CO to other compounds in vivo proceeds at a negligible rate (24).

Whether CO may in addition arise from other sources totally unrelated to heme metabolism is unknown. A small fraction of endogenous CO appears to arise from sources other than circulating hemoglobin, however, since $\dot{V}co$ exceeds rates of circulating heme catabolism by 20–26% in both normal subjects and patients with hemolytic anemia. We have therefore investigated the possibility that this "excess CO" might be related to the "early labeled" peak of stercobilin, and have attempted to quantitate such excess CO in patients who excreted increased amounts of "early labeled" stercobilin.

We have found that $\dot{V}co$ in such patients greatly exceeds turnover of circulating heme as calculated from ^{51}Cr erythrocyte survival studies, in contrast to normal individuals and subjects with hemolytic anemia. Using glycine-2-^{14}C as a precursor for heme, we have found also that an "early labeled" peak of CO accompanies the "early labeled" peak of stercobilin, and that this "early labeled" CO is increased in patients with ineffective erythropoiesis.

Methods

Subjects. Subjects with normal erythropoiesis included a healthy adult male volunteer and an elderly woman with a chronic leukopenia of unknown etiology. Marrow aspirate from the latter showed normal erythroid maturation and normal iron stores, and karyotypic analysis of marrow mitotic figures was normal.

Subjects with abnormal erythropoiesis included one patient with thalassemia, and four patients with primary refractory anemia. M.L., the thalassemic patient, of Italian ancestry, had typical morphologic changes in the peripheral blood, and hemoglobin electrophoresis pattern was 83% A, 11.1% A_2, and 5.9% F. Study of the family could not be carried out and it is not known whether the patient is a homozygote or heterozygote. This subject was studied on two separate occasions, before splenectomy for hypersplenism, and approximately 1 yr later. The four patients with refractory anemia were chronically anemic, had no apparent underlying disease, and showed no response to therapeutic trials of pyridoxine, folic acid, and oral crude liver extract. Patients A.H. and E.M. had many abnormal "ringed sideroblasts" in the marrow, but patients D.A. and F.F. did not. Erythroid hyperplasia was present in all five patients with anemia and all showed increased iron stores in marrow aspirates. Maturation was normoblastic in four and megaloblastic in one (F.F.). This patient, a mentally retarded eunochoid male who had been anemic since infancy, had a low serum folate activity by *L. casei* assay (25) but showed no hematologic response to oral folic acid therapy. Serum vitamin B_{12} levels by *E. gracilis* assay (26) were normal in all patients and none excreted methylmalonate in the urine (27).

Studies of erythrokinetics and carbon monoxide production were also carried out in a patient with porphyria cutanea tarda (A.V.), who had a bullous skin eruption, with hyperpigmentation and hirsutism, and hepatosplenomegaly. Bromsulphalein retention was 17% at 45 min. Urinary excretion values were porphobilinogen 0.4–2.5 mg/24 hr, uroporphyrin 3.3–6.6 mg/24 hr, and coproporphyrin 0.96–3.1 mg/24 hr. Fecal excretion values were uroporphyrin 1.5 mg/g of dry stool, coproporphyrin 4.0 mg/g, and protoporphyrin 8 μg/g. This patient was not anemic but sternal aspirate showed normoblastic erythroid hyperplasia and increased storage iron.

All subjects were clinically stable and thought to be in a steady state hematologically at the time of study. Pertinent clinical and laboratory data are given in Table I.

Procedures. Routine hematologic studies were done by standard techniques. Serum iron and iron-binding capacity were determined by the method of Ramsey (28).

TABLE I

Clinical and

Patient	Age/Sex	Wt	Diagnosis	Hb	Reticulocytes	Serum bilirubin direct/total
	yr	*kg*		*g/100 ml*	%	*mg/100 ml*
M.L.	41 F (1964)	52.0	Thalassemia	5.5	3.0	0.14/2.2
	42 F (1965)	52.0	(Post splenectomy)	6.8	10.6	0.11/2.0
D.A.	68 M	54.0	Primary refractory anemia	7.0	1.0	0.18/0.86
F.F.	18 M	59.0	Primary refractory anemia	6.2	1.0	0.02/0.36
E.M.	84 F	43.2	Refractory sideroblastic anemia	6.5	2.3	—/0.94
A.H.	67 F	72.8	Refractory sideroblastic anemia	6.7	3.0	0.09/0.16
A.V.	51 F	50.4	Porphyria cutanea tarda	13.9	2.0	0.15/0.71
G.B.	68 F	66.6	Leukopenia of unknown etiology	12.0	1.9	—/0.50
M.D.	35 M	72.7	Normal volunteer	14.5	0.6	0.08/0.33
Normal values§						

* Iron-binding capacity.
‡ Plasma iron transport rate.
§ Normal range, this laboratory.

Clearance and reappearance of ^{59}Fe were studied by the method of Loeffler et al. (29) and plasma iron transport rate (PITR) was calculated as milligrams of iron cleared per 100 ml of plasma per day. Blood volume and red cell survival time were determined by the radiochromate method of Read (30) using acid citrate–dextrose anticoagulant. Total fecal urobilinogen excretion was determined in pooled collections by the method of Schwartz, Sborov, and Watson (31) and the results of several separate collections were averaged. Resting blood carboxyhemoglobin ([COHb]) and endogenous carbon monoxide production (Vco) were determined by previously described methods (32, 23).

Isotope studies. ^{14}C-labeled glycine [1] was administered intravenously over a period of 30 sec in a dose of 100 μc and determinations were made of the subsequent incorporation of ^{14}C into circulating erythrocyte heme, plasma bilirubin, fecal stercobilin, and endogenous carbon monoxide.

Heme was isolated from peripheral blood as protoporphyrin methyl ester by the method of Grinstein (33) and recrystallized four times.

Bilirubin was isolated from plasma by a modification of the method of Ostrow, Hammaker, and Schmid (34), with unlabeled carrier. Total bilirubin concentration was determined by the method of Malloy and Evelyn (35) on an aliquot of heparinized venous plasma. To 10–20 ml of plasma were added 100 mg of ascorbic acid and 5 mg of recrystallized, unlabeled bilirubin [2] dissolved in 2 ml of 0.03 M phosphate buffer (pH 11.3) containing 15% bovine albumin.[3] The pH was adjusted to 4.0 with acetic acid, and the sample was shaken with 5 volumes of methanol for 10 min. 10 volumes of chloroform were added, and the sample was shaken for an additional 20 min. The whitish residue was then reextracted twice more in the same manner. The combined methanol-chloroform extracts were then washed and the bilirubin isolated according to Ostrow et al. (34). Yields were in the range of 20–40%. Specific activity remained constant after the third recrystallization.

Stercobilin was isolated from stool collections by Watson's method as modified by Gray and Scott (3) and was recrystallized three times before counting.

For radioassay of ^{14}C-labeled protoporphyrin methyl ester, bilirubin, and stercobilin, samples were weighed and then combusted to CO_2 in a Schoeninger flask using the method of Nathan, Gabuzda, and Gardner (36). Ethanolamine and methoxyethanol (1:2 mixture) were added

[1] Glycine-2-^{14}C, 2.31 $\mu c/\mu$mole, New England Nuclear Corp., Boston, Mass.

[2] Fisher Scientific Company, Fair Lawn, N. J.

[3] Pentex Inc., Kankakee, Ill.

TABLE I

laboratory data

Serum iron/IBC*	^{59}Fe $t_{\frac{1}{2}}$	PITR‡	Maximal ^{59}Fe reappearance	^{51}Cr $t_{\frac{1}{2}}$	Fecal urobilinogen
μg/100 ml	*min*	*mg/100 ml plasma per day*	*%*	*days*	*mg/day*
69/162	14	4.4	15	14.5	470
140/240	25	5.6	27	10	495
74/216	94	0.8	65	18	17
200/240	82	2.4		21	35
164/345	75	2.2	55	11	17
200/200	45	4.4	24	14	27
202/342	107	1.9	80	27	
90/285	60	1.5	99	28	51
100/333					18
70–200/250–400	60–120	0.8–1.5	80–100	25–30	

to absorb CO_2 and an aliquot was mixed with scintillator solution [4] for counting in a well-type scintillation spectrometer.[5] Toluene-^{14}C was used as internal standard; counting efficiency was 30–40%.

Carbon monoxide was collected with a washout procedure similar to that previously described (21), by increasing the O_2 partial pressure in the rebreathing system to 80–90% and collecting expired air in a large rubber bag (100 liter capacity). Up to 50% of the total body CO pool could be collected over a 40 min period. The bag contents were subsequently drawn through a train with the following components: (*a*) Ascarite [6] for CO_2 absorption; (*b*) an alkali check trap to detect incomplete removal of CO_2; (*c*) a column of 0.1 M palladium chloride in 0.06 N HCl, to oxidize CO to CO_2 (length of column 5 m, total volume of solution 100 ml); (*d*) Two or three alkali CO_2 traps in series; and (*e*) a needle valve flow regulator connected to a vacuum source. The conical collecting tubes of the alkali CO_2 traps and the check trap contained 3 ml of 0.1 N NaOH. The system had a trapping efficiency for CO of 50–60% for flow rates of 10–20 ml/min, at the low partial pressures of CO encountered. The CO_2 trapped was quantitated by titration of residual alkali against 0.2 N HCl to pH 8.5, using a pH meter [7] and syringe microburet.[8] After dilution to standard volume, a 0.5 ml aliquot was mixed with scintillator solution,[9] and counted for radioactivity in the liquid scintillation spectrometer. In some collections where specific activity was low, larger aliquots were mixed with 19 volumes of ethanol and distilled to dryness under reduced pressure. The residue was redissolved in 0.5 ml of H_2O, mixed with scintillator solution, and counted.

Later studies measured blood ^{14}CO content directly by an adaptation of the ferricyanide method for COHb determination (32). Heparinized venous blood (10–40 ml) was transferred to a 100 ml reaction flask. N_2 was bubbled through the sample and drawn into an evacuated 500 ml Erlenmeyer flask ($PdCl_2$ flask) which contained 10 ml of 3.39×10^{-4} M $PdCl_2$ in 8×10^{-3} N HCl and was fitted with a center well containing 5 ml of 1 N NaOH. A calculated excess of 0.7 M potassium ferricyanide was then added to the blood sample to oxidize hemoglobin to methemoglobin and thereby release CO. The gas phase of the reaction flask was again flushed with N_2 into a second $PdCl_2$ flask. As in the washout experiments with expired air, the system included an Ascarite CO_2 absorber followed by an alakali check trap. After allowing the $PdCl_2$ flasks to stand for 7–10 days at 24°C, we determined the residual $PdCl_2$ spectrophotometrically by the method of Allen and Root (37) to quantitate the CO converted to CO_2. The contents of the center well were transferred to a second reaction flask and excess H_2SO_4 added to liberate CO_2 which was drawn into a trap containing ethanolamine and methoxyethanol (5 ml of a 1:2 mixture). This was then added directly to scintillator solution [10] and the radioactivity determined. Negligible radioactivity was detected in the first $PdCl_2$ flask, before adding ferricyanide to the blood sample. Control experiments with large amounts of ferricyanide added to ^{14}C labeled hemoglobin demonstrated no production of ^{14}CO from in vitro degradation of hemoglobin in this system. The determination of CO had a standard deviation of $\pm$ 0.09 μmole by this method, which represented 4.0–18% of CO in the blood specimens analyzed.

TABLE II

*Comparison of methods for determining ^{14}CO**

Method	Specific activity	
	Day 2	Day 3
	dpm/μmole	
Washout $PdCl_2$	56	140
Blood $PdCl_2$	66	157
Ionization chamber	—	158

* Simultaneous determinations on patient M.L.

Simultaneous measurements of ^{14}CO specific activity were carried out in patient M.L. to compare these two methods with each other and with a third, previously described method (21) utilizing an ionization chamber to measure radioactivity in the rebreathing system gas. As shown in Table II, there was good agreement between these different methods for determining ^{14}CO specific activity. These results also indicate that ^{14}CO was the only radioactive gas present in the rebreathing system, since specific activity by the third method should be appreciably higher if another ^{14}C-containing gas were present in significant amounts. Furthermore, by lowering the partial pressure of O_2 in the rebreathing system, it was possible to show that total ^{14}C activity per 1000 ml fell in proportion to the change in oxygen tension, while specific activity remained constant (Table III). This finding is also consistent with the result to be expected if all the radioactivity present in the rebreathing system gas was due to ^{14}CO.

Analytic procedures. The rate of catabolism of circulating erythrocyte hemoglobin was calculated as equal to total circulating hemoglobin divided by mean red cell survival time. Total circulating hemoglobin was taken as the

[4] Toluene: methoxyethanol (2:1 mixture) containing 4 g/liter 2,5-diphenyloxazole (PPO), Packard Instrument Co., Inc., Downers Grove, Ill.

[5] Tri-Carb liquid scintillation spectrometer, Packard Instrument Co., Inc., Downers Grove, Ill.

[6] Arthur H. Thomas, Co., Philadelphia, Pa.

[7] Model 76 pH meter, with No. 39166 electrode probe assembly, Beckman Instruments, Inc., Fullerton, Calif.

[8] Model SB2, Micrometric Instrument Co., Cleveland, Ohio.

[9] Toluene: ethanol (1.68:1 mixture) containing 2.5 g/liter 2,5-diphenyloxazole (PPO) and 31 mg/liter 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), Pilot Chemicals, Inc., Watetown, Mass.

[10] Toluene: methoxyethanol (2:1 mixture) containing 4.0 g/liter 2,5-bis [2(5-*tert*-butylbenzoxazolyl)]-thiophene (BBOT). Packard Instrument Co., Inc., Downers Grove, Ill.

TABLE III

*Effect of changing oxygen tension**

O_2‡	Total radioactivity§	Specific activity CO‖
%	*dpm/1000 ml*	*dpm/μmole*
87	5.95×10^2	156
52	3.24×10^2	159

* Successive studies in patient M.L. on day 3 after glycine-2-^{14}C.

‡ O_2 partial pressure in rebreathing system gas.

§ Rebreathing system gas activity measured by ionization chamber method.

‖ Total radioactivity divided by CO concentration in rebreathing system gas, determined by infrared analyzer method (32).

product of peripheral hemoglobin concentration and ^{51}Cr blood volume assuming a value of 0.91 for the ratio of "whole body hematocrit" to peripheral venous hematocrit (38). To determine mean red cell survival time, we corrected the RBC ^{51}Cr activity data for elution at an assumed constant rate of 1.29%/day (39, 23) and plotted them on rectangular coordinate paper. In two instances (patients M.L. and E.M.) the resulting curve of ^{51}Cr activity was not linear with time, and the initial slope was extrapolated to the time axis and this intercept taken as mean survival time (40). In the other patients the data corrected for elution showed a linear decrease in activity with time, and a regression line was plotted by the method of least squares; its intercept with the time axis was then taken as the mean red cell survival time. In the normal subject M.D., protoporphyrin-^{14}C specific activity was graphed vs. time, and the interval from 50% of maximal activity on the ascending limb of the curve to 50% of maximal on the descending limb was taken as mean survival time.

Heme catabolism (Vheme) was calculated in micromoles per hour by use of the following formula:

$$\dot{V}\text{heme } (\mu\text{moles/hr}) = \frac{\text{g Hb catabolized/day}}{0.017 \text{ g}/\mu\text{mole}} \times 1/24$$

$$= 2.45 \times \text{g Hb catabolized/day.}$$

CO production not attributable to heme catabolism, so-called "excess Vco," was defined as the difference in μmoles/hour between measured Vco and Vheme.

In patients whose blood ^{14}CO was measured directly, total ^{14}CO production during the "early labeled" peak was calculated from the specific activity of endogenously produced ^{14}CO. Since the body CO pool is composed of both endogenous CO and unlabeled exogenous CO, blood ^{14}CO specific activity was corrected for this dilution effect, by assuming an exogenous carboxyhemoglobin concentration of 0.40% (41). Blood ^{14}CO specific activity was multiplied by the correction factor [COHb]/([COHb] − 0.40%), where [COHb] is the measured blood carboxyhemoglobin concentration in per cent saturation, to determine specific activity of endogenously produced ^{14}CO, and the latter was then graphed vs. time, over the arbitrary interval of 6 days after isotope administration. The area under this curve, in units of disintegrations/minute per micromole × days, was multiplied by Vco (in micromoles/day) and the product (in distintegrations/minute) was taken to equal "early ^{14}CO" production. This calculation assumes a constant Vco and a constant total body CO pool, assumptions which seem justifiable on the basis of previous studies in other subjects (41).

Total ^{14}C incorporation into circulating heme after isotope administration was calculated as the product of maximal specific activity of heme (isolated as protoporphyrin methyl ester) and the quantity of total circulating heme. Since one of the eight ^{14}C carbon atoms of heme which has been biosynthetically labeled with glycine-2-^{14}C is available for conversion to CO (42), "potential ^{14}CO" available from catabolism of circulating heme has been calculated as one-eighth of total heme ^{14}C.

Statistical evaluations were performed using standard methods as outlined by Dunn (43).

Results

^{59}Fe and ^{51}Cr studies and bile pigment excretion. Ferrokinetic studies and ^{51}Cr-RBC survival data are listed in Table I. Of the patients with anemia, the plasma iron transport rate was elevated in all except one (D.A.), whereas ^{59}Fe reappearance was subnormal in all. ^{51}Cr half time was abnormally short in all patients with anemia, but normal in the patient with porphyria cutanea tarda (A.V.).

Fecal urobilinogen excretion values were quite variable among different collections from the same patient. The mean values (listed in Table I) were below the range usually accepted as normal in all except the thalassemic patient, M.L. The hemolytic index (44) was slightly elevated in patient F.F., however, and markedly elevated in patient M.L.

Heme catabolism and CO production. Data for Vheme and Vco are shown in Table IV. The rate of catabolism of circulating erythrocyte hemoglobin varied between 1.67 and 9.5 g/day, equivalent to 4.1–23.3 μmoles of heme/hr. Carboxyhemoglobin saturation ([COHb]) was in the normal range in two of the patients with anemia, despite elevated Vco values. Vco was markedly elevated in patients M.L. and A.V., but was not significantly elevated in patient F.F.

Vco was greater than Vheme in all patients with anemia. The highest value for "excess Vco" was seen in patient A.V. with porphyria. The mean Vco/Vheme ratio for the group of five patients with ineffective erythropoiesis was 3.0 ± 0.6 (SE).

TABLE IV

*Hemoglobin catabolism and CO production data**

Patient	Mean RBC survival time	Total circulating Hb	V̇heme	[COHb]	V̇co	V̇co/V̇heme	"Excess V̇co"	"Excess V̇co" / Total V̇co
	days	*g*	*μmoles/hr*	*% saturation*	*μmoles/hr*		*μmoles/hr*	
M.L. (1964)	24.5	230	23.3	2.64	94.2	4.1	70.9	0.75
(1965)	26.0	226	21.2	2.27	64.7	3.1	43.5	0.67
D.A.	49.0	198	10.0	0.72	25.0	2.5	15.0	0.60
F.F.	80.0	134	4.1	1.19	20.5	5.1	16.4	0.80
E.M.	22.5	193	21.1	0.90	27.5	1.3	6.4	0.23
A.H.	34.0	223	16.0	1.15	37.6	2.4	21.6	0.58
G.B.	80.0	450	13.8	0.80	19.9	1.4	6.1	0.31
M.D.	124.0‡	726§	14.4§	0.76	14.5	1.0	0.1	0.01
A.V.	93.0	363	9.6	1.97	88.0	9.2	78.4	0.89
Normal values (18, 32) (mean ±SD)				0.88±0.11	18.7±3.18	1.27±0.26	4.0±3.7	0.21±0.14

* V̇heme, rate of circulating heme catabolism; [COHb] venous blood carboxyhemoglobin; V̇co, rate of CO production; "Excess V̇co," V̇co minus V̇heme.
‡ From protoporphyrin-^{14}C data.
§ Assumed blood volume of 70 ml/kg.

Studies with glycine-2-^{14}C. All patients studied for ^{14}CO showed the prompt appearance of an "early labeled" peak of ^{14}CO after glycine-2-^{14}C administration. Figs. 1 and 2 show the values obtained by the washout technique, with stercobilin specific activities for comparison. The normal volunteer, M.D., had significant amounts of ^{14}CO in the body pool over the first 4 days after glycine-2-^{14}C, but negligible amounts of ^{14}CO were present when he was next studied on day 9. ^{14}CO later reappeared concomitantly with labeled stercobilin as the cohort of circulating ^{14}C-labeled red cells was destroyed at the end of their normal life span.

Fig. 3 shows the incorporation of ^{14}C into circulating heme and fecal stercobilin in those subjects whose ^{14}CO collections are depicted in the bar graphs of Figs. 1 and 2, and also in patient D.A., whose ^{14}CO activity was not studied. In comparison to the normal subject, M.D., the three anemic patients show a marked elevation of specific activity of the "early labeled" stercobilin. Plasma bilirubin activity was determined in patient M.L. and is seen to parallel stercobilin activity closely.

Fig. 4 shows similar curves of isotope incorporation in the studies where ^{14}CO was determined by the direct blood ^{14}COHb method. Compared to patient G.B. with normal erythropoiesis, the two patients with sideroblastic anemia (A.H. and E.M.) show markedly increased incorporation of isotope into stercobilin and into CO, with

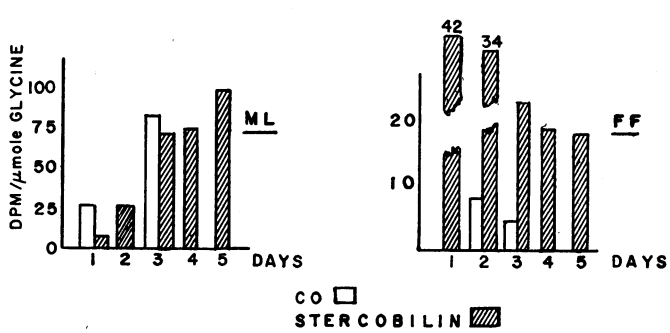

FIG. 1. ^{14}C INCORPORATION INTO CO AND STERCOBILIN. Specific activity of CO collected by washout technique, and of fecal stercobilin, in patients with ineffective erythropoiesis, on days 1–5 after glycine-2-^{14}C administration. Activity is graphed as dpm/μmole of glycine, assuming seven labeled carbon atoms per molecule of stercobilin are derived from precursor glycine (42).

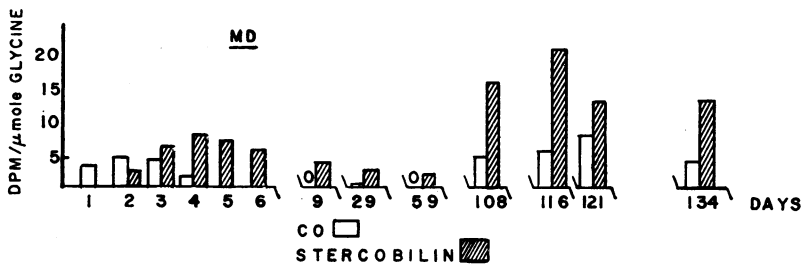

FIG. 2. ^{14}C INCORPORATION INTO CO AND STERCOBILIN. Specific activity of CO collected by washout technique, and of fecal stercobilin in a normal subject, after glycine-2-^{14}C administration.

activities that are comparable to those seen in the thalassemic patient (M.L.). Plasma bilirubin specific activity paralleled that of CO and stercobilin in patients A.H. and E.M. Bilirubin activity in the second study of patient M.L. was significantly lower than that of the corresponding stercobilin, however. Plasma bilirubin radioactivity in patient G.B. was too low to determine specific activity.

Table V shows calculated "early ^{14}CO" production over the 6 days after isotope administration for the four patients studied by the direct blood $^{14}COHb$ method. The thalassemic M.L. shows an "early ^{14}CO" production which is 13.8 times that of the patient with normal erythropoiesis, G.B. The two patients with refractory sideroblastic anemia (E.M. and A.H.) had values which were 5.7 and 12 times that of G.B.

The incorporation of ^{14}C from glycine into circulating heme is also shown in Table V. The lowest incorporation (0.45% of administered dose) is shown by patient F.F., with primary refractory anemia. The other patients with refractory anemia showed variable lowering of ^{14}C incorporation into heme as compared to the two normals, but the thalassemic, M.L., showed an apparently normal production of circulating ^{14}C heme on both occasions.

The sum of "early ^{14}CO" and "potential ^{14}CO" available from the eventual catabolism of labeled circulating heme, is shown in Table V as "total ^{14}CO." The ratio of "early ^{14}CO" to "total ^{14}CO" was strikingly high (0.49–0.76) in the three patients with anemia as compared to the value (0.10) in patient G.B., with normal erythropoiesis.

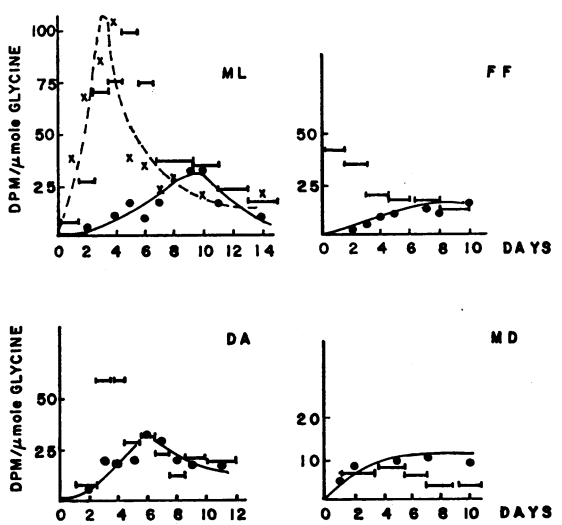

FIG. 3. ^{14}C INCORPORATION INTO HEME, STERCOBILIN, AND BILIRUBIN. Specific activity is shown vs. time after administration of glycine-2-^{14}C, in three patients with ineffective erythropoiesis and in a normal volunteer (M.D.). Heme, solid circles; stercobilin, bars (indicating periods of collection); bilirubin (isolated in patient M.L. only), X's. Note scale of specific activity is different for M.D.

Discussion

The patients with anemia in this report showed a major element of ineffective erythropoiesis, with normal or increased plasma ^{59}Fe turnover but subnormal ^{59}Fe reappearance, and exaggerated "early labeling" of bile pigment after glycine-2-^{14}C administration. The ^{51}Cr studies in these patients also demonstrated a significant shortening in the survival of circulating erythrocytes, with half life as short as 10 days in the thalassemic patient, M.L. Although this hemolysis undoubtedly led to some "early" excretion of labeled bile pigments by destruction of cells newly released into the circulation, the pattern of stercobilin label-

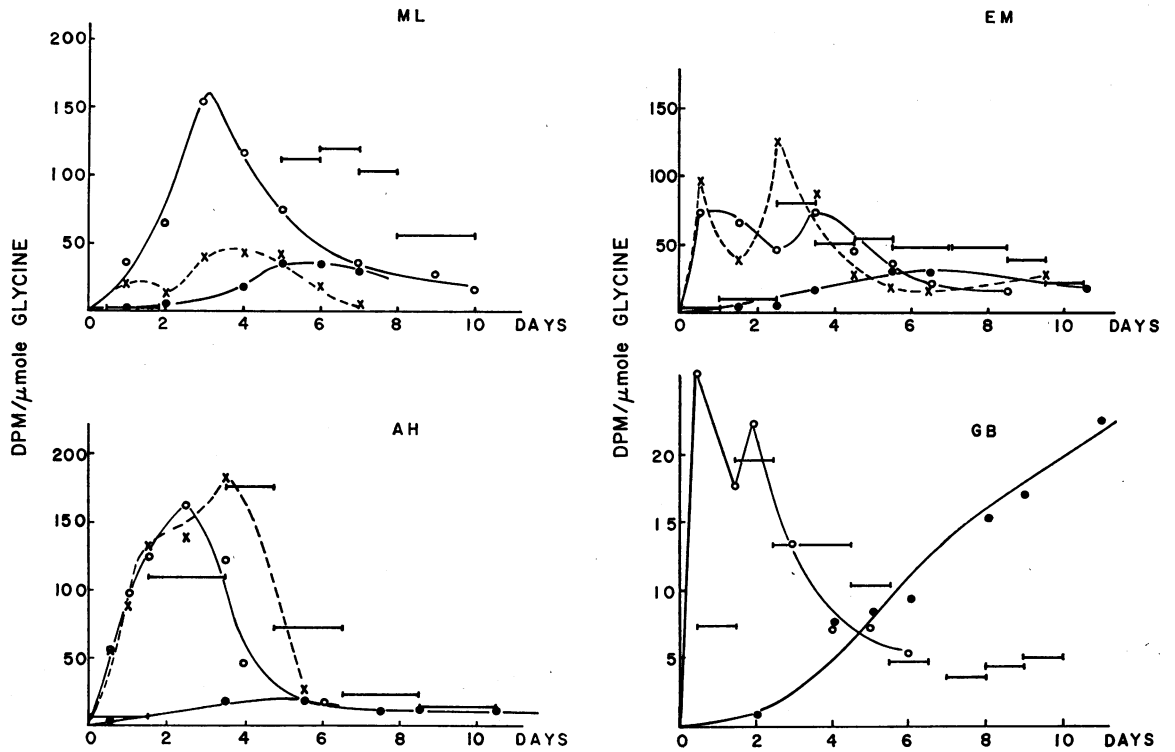

FIG. 4. ^{14}C INCORPORATION INTO HEME, STERCOBILIN, BILIRUBIN, AND CO. Specific activity is shown vs. time after administration of glycine-2-^{14}C, in three patients with ineffective erythropoiesis and in a patient with normal erythropoiesis (G.B.). Heme, solid circles; stercobilin, bars (indicating periods of collection); bilirubin, X's; CO (isolated from blood COHb), open circles. Note scale of specific activity is different for G.B.

ing was clearly different in these patients from that described for typical hemolytic states such as sickle cell anemia (45) or hereditary spherocytosis (16). Maximal stercobilin activity in the "early labeled" peak of our patients preceded rather than followed maximal activity of circulating heme, and maximal specific activity of stercobilin exceeded that of heme by several fold.

TABLE V

^{14}C isotope data

Patient	"Early ^{14}CO"*	Max. heme sp. act.‡	Total circulating ^{14}C-heme§	Incorp. of glycine-2-^{14}C into heme‖	"Potential ^{14}CO"¶	"Total ^{14}CO"**	"Early ^{14}CO" / "Total ^{14}CO"
	dpm	*dpm/μmole*	*dpm*	%	*dpm*	*dpm*	
M.L. (1964)		305	4.5×10^6	2.0			
(1965)	8.80×10^5	282	4.1×10^6	1.8	5.1×10^5	1.39×10^6	0.63
D.A.		276	3.5×10^6	1.57			
F. F.		114	9.9×10^5	0.45			
E.M.	3.63×10^5	242	3.0×10^6	1.35	3.75×10^5	7.38×10^5	0.49
A.H.	7.72×10^5	134	1.93×10^6	0.87	2.41×10^5	1.01×10^6	0.76
G.B.	6.40×10^4	158	4.6×10^6	2.1	5.75×10^5	6.39×10^5	0.10
M.D.		91	4.3×10^6	1.9			

* Total ^{14}CO production during days 0–6 after glycine-2-^{14}C.
‡ Maximal specific activity of circulating heme, isolated as protoporphyrin methyl ester.
§ Total circulating heme (μmoles) times maximal heme specific activity.
‖ Total circulating ^{14}C-heme divided by 2.2×10^6 dpm.
¶ Total circulating ^{14}C-heme times 1/8.
** "Early ^{14}CO" plus "potential ^{14}CO."

These results are essentially the same as those previously reported by others for patients with thalassemia (12) and primary refractory anemia (4, 13).

Analysis of CO metabolism also showed a significant difference between these patients with ineffective erythropoiesis and patients with straightforward hemolytic anemia. In the latter group a close correlation has been previously shown to exist between CO production and circulating heme turnover, with a reported mean $\dot{V}co/\dot{V}heme$ ratio of 1.40 ± 0.1 (SE), which is only slightly higher than the calculated mean ratio of 1.27 found in normal subjects (23). The mean $\dot{V}co/\dot{V}heme$ ratio in the present group of patients with anemia was 3.0 ± 0.6 (SE). The difference between this value and the mean for patients with hemolytic anemia is highly significant ($P < 0.01$). In four of the five anemic patients in the present study, the ratio of "excess $\dot{V}co$" to total $\dot{V}co$ was greater than 0.5, implying that over 50% of endogenously produced CO in these cases originated from sources other than circulating heme, as compared to a mean of 26% from such sources in patients with hemolytic anemia.

It is apparent that the errors involved in measuring $\dot{V}heme$ from ^{51}Cr data are inherent in calculating "excess $\dot{V}co$" as well as $\dot{V}co/\dot{V}heme$. Such errors may be quite sizable, due to variability in elution rates of ^{51}Cr and the problem of detecting minor populations of circulating red cells with very brief half lives. Similar difficulties exist in calculating $\dot{V}heme$ in patients with hemolytic anemia, however, so that the comparison between $\dot{V}co/\dot{V}heme$ ratios in the two groups should remain valid. In particular, it seems very unlikely that variation in ^{51}Cr elution is responsible for the high $\dot{V}co/\dot{V}heme$ ratios found here. If we assume an elution rate of 0.62%/day, the lowest value observed by Cline and Berlin (39), the mean $\dot{V}co/\dot{V}heme$ ratio for the five patients with ineffective erythropoiesis is 2.25, still significantly greater ($P < 0.05$) than the mean for patients with hemolytic anemia.

It should be pointed out that total circulating hemoglobin in the present series of patients has been calculated from blood volumes determined by the ^{51}Cr method, in contrast to the previously reported series of patients with hemolytic anemia, where blood volumes were determined by Sjöstrand's CO method (46). Although estimates of total hemoglobin by the latter method are approximately 16% higher than those obtained from ^{51}Cr labeling (47), recalculating $\dot{V}heme$ on this assumption gives a mean $\dot{V}co/\dot{V}heme$ ratio of 2.54 in the patients with ineffective erythropoiesis.

These data are entirely consistent with the additional finding that the patients with ineffective erythropoiesis showed markedly increased incorporation of isotope into "early labeled" ^{14}CO, which appeared simultaneously with "early labeled" bilirubin, and preceded the maximal incorporation of isotope into circulating heme. It is evident that much of the "excess $\dot{V}co$" generted by these patients can be attributed to this early labeled fraction, and that carbon monoxide metabolism parallels bile pigment metabolism in reflecting ineffective erythropoiesis as well as catabolism of circulating hemoglobin.

The studies in the two subjects with normal erythropoiesis, M.D. and G.B., demonstrate that the "early CO" peak, like the early stercobilin peak, is also seen in normal man. The similar appearance of an "early labeled" peak of CO in rats after glycine-2-^{14}C administration has also been reported by Landaw and Winchell (48). The difference of 20% between $\dot{V}co$ and $\dot{V}heme$ reported in normal humans can be partially attributed to this "early labeled" CO peak, although the "early ^{14}CO" produced in G.B. represented only 10% of her calculated total ^{14}CO as shown in Table V, and in M.D. the early peak was 6.5% of total ^{14}CO, determined from the relative areas under the early and late peaks.

If all "excess $\dot{V}co$" were due to the "early labeled" peak of CO, then there should be close correlation between the two ratios, excess $\dot{V}co$/total $\dot{V}co$, and early ^{14}CO/total ^{14}CO. The presence of significant hemolysis of circulating red cells, as demonstrated by ^{51}Cr studies in patients M.L., A.H., and E.M., makes the interpretation of the latter ratio hazardous, however. The destruction of erythrocytes newly released into the circulation, within the first 6 days after glycine administration, would cause an increase in "early ^{14}CO," and a high early ^{14}CO/total ^{14}CO ratio. Nevertheless, as can be seen from Tables IV and V, the two ratios are quite similar to each other for patient M.L. (0.67 and 0.63) and for patient A.H. (0.58 and 0.76).

Hemolysis of newly released red cells would also tend to lower the maximal specific activity of circulating heme, and lead to underestimation of the incorporation of glycine-2-^{14}C into circulating heme. The thalassemic patient M.L., for example, in all likelihood had a supranormal incorporation of glycine-2-^{14}C into circulating heme rather than the values calculated and shown in Table V. This finding is in marked contrast to that reported by Grinstein et al. in their patients with thalassemia major (12), where the per cent of glycine incorporated varied between 0.04% and 0.16%. If we assume that all ^{14}CO produced by patient M.L. arose from the catabolism of labeled hemoglobin, total glycine incorporation into heme is 5.1%, well above the findings in normals.

While the "early labeled" bile pigment, particularly in patients with ineffective erythropoiesis, has been attributed to turnover of hemoglobin in the marrow, it is evident that many other heme compounds in the body can serve as precursors for bile pigment. Conversion of free hemin and free protoporphyrin to bile pigment has been demonstrated in experimental animals (49–52), and we have similarly recovered ^{14}CO after the injection of hemin-^{14}C and protoporphyrin-^{14}C into dogs (21). In addition, recent findings in several laboratories (6–9) have implicated the liver in the production of bilirubin de novo from glycine and delta amino levulinic acid, and have suggested that turnover of heme compounds in the liver may give rise to a significant fraction of the early labeled peak of stercobilin. Studies with rat liver homogenate preparations have demonstrated the in vitro conversion of glycine-2-^{14}C to ^{14}CO and bilirubin-^{14}C (53) so that a portion of the "early labeled" peak of CO undoubtedly arises from hepatic sources as well. In the presence of increased marrow erythropoietic activity, the contribution of the liver to total heme synthesis presumably would be relatively quite small, but the possibility has not been excluded that there is an increase in hepatic heme turnover in patients with ineffective erythropoiesis. As shown in Fig. 4, the specific activity curves for ^{14}CO or bilirubin-^{14}C were biphasic in several patients, supporting the suggestion of Yamamoto and associates that two components can be distinguished temporally in "early labeled" bilirubin (6). Both the first component, which has been attributed to the liver, and the second component attributed to the marrow, appear to be increased in the bilirubin-^{14}C curves for patients E.M. and A.H.

Data presented by Schmid (54) have indicated that the microsomal enzymes, cytochrome P-450 and cytochrome b_5, are the major hepatic hemoproteins involved in early labeling of bilirubin, with minor contributions arising from turnover of catalase and tryptophane pyrrolase. Induction of the microsomal cytochromes by exposure to barbiturates or other drugs (55) may greatly augment this pathway of bile pigment production, and significant elevations of $\dot{V}_{CO}$ have in fact been observed in normal subjects receiving barbiturates (R. F. Coburn, unpublished observations). None of the patients with ineffective erythropoiesis in this report were on barbiturates or other drugs known to induce microsomal cytochromes at the time of their studies, however, and none had clinically apparent liver disease.

The liver does appear to be the source of excessive porphyrin production in porphyria cutanea tarda, and the extremely high $\dot{V}_{CO}$ and $\dot{V}_{CO}/\dot{V}_{heme}$ ratio found in the porphyria patient, A.V., were of considerable interest in this regard. Only 12% of her $\dot{V}_{CO}$ could be attributed to circulating heme catabolism. It seems quite possible that a significant portion of her "excess $\dot{V}_{CO}$" may have arisen from porphyrin compounds in her liver. Isotopic studies in patients with porphyria cutanea tarda (3, 56) have not demonstrated an increased "early labeled" peak of stercobilin, however, and an investigation of the "early ^{14}CO" peak in such patients would obviously be highly desirable.

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