“Early-Peak” Carbon Monoxide Production in Certain Erythropoietic Disorders

By McDonald K. Horne, III, Wendell F. Rosse, Edward G. Flickinger, and Herbert A. Saltzman

The “early-labeled” peak (ELP) of \(^{14}C\)O excretion following injection of glycine-2-\(^{14}C\) was used to study erythropoiesis in a patient with sideroblastic anemia and in four subjects with myeloproliferative disorders. The ELP was greatly enlarged in all patients, as compared with a normal volunteer. The contour of the peaks from the hematologically abnormal subjects suggested the presence of increased erythroid heme degradation. In the patient with sideroblastic anemia, all hours of the early peak were significantly reduced after transfusion. This was interpreted to mean that even the earliest or “nonerythroid” phase of the peak is influenced by erythropoietic activity, at least under conditions of erythropoietic stress.

When isotopes of the heme precursor glycine are injected into mammals, labeled heme catabolites (bilirubin, urobilin, and carbon monoxide) appear in the blood, stool, and breath.\(^1\)\(^-\)\(^5\) An early-labeled peak (ELP) of catabolite excretion occurs during the first week after the administration of the glycine, while a late-labeled peak (LLP) normally appears 3–4 mo later.\(^6\) The late peak clearly originates from the degradation of the heme of circulating hemoglobin, since it coincides with the disappearance of isotope from the circulating red cells.

Normally, the early peak represents 10%–20% of the total catabolite excretion.\(^1\)\(^\)\(^2\)\(^\)\(^4\) Its size, however, varies with the erythroid cellularity of the bone marrow. The ELP is abnormally small in aplastic anemia,\(^6\) for instance, and enlarged under conditions of erythroid hyperplasia, such as in thalassemia\(^7\) and pernicious anemia,\(^8\) and following experimental hemorrhage.\(^9\) This correlation has been interpreted to mean that the early peak arises from erythropoietic activity. However, several studies using experimental animals have shown that the earliest hours of the peak are relatively insensitive to erythropoietic stimulation or suppression,\(^5\)\(^10\) while a later phase is augmented by hypoxia or hemorrhage\(^10\) and diminished by radiation-induced bone marrow damage.\(^5\) A biphasic nature of the ELP in humans has also been found,\(^11\)\(^12\) although the differential responsiveness to alterations in erythropoiesis has not been demonstrated.

The current study was undertaken to investigate the characteristics of the ELP in man. Glycine-2-\(^{14}C\) was used as a heme precursor and injected into experimental subjects, whose breath was subsequently analyzed for the heme catabolite \(^{14}CO\). During the degradation of heme-\(^{14}C\), the alpha-methene carbon, which is labeled by the glycine-2-\(^{14}C\), is removed from the protoporphyrin ring to form \(^{14}CO\).\(^13\)\(^14\) Since the half-time of CO excretion is only a few hours,\(^15\)
Table 1. Clinical Data

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age/Sex</th>
<th>Wt (kg)</th>
<th>Hb g/100 ml</th>
<th>Retic. count (%)</th>
<th>Chromium-51 t½ (days)</th>
<th>Bone Marrow</th>
<th>Spleen*</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.H.</td>
<td>67/M</td>
<td>55.5</td>
<td>13.7</td>
<td>1.2</td>
<td>29</td>
<td>Normal</td>
<td>NP</td>
<td>Normal</td>
</tr>
<tr>
<td>H.H.</td>
<td>65/F</td>
<td>69.7</td>
<td>8.0</td>
<td>3.2</td>
<td>30</td>
<td>Ringed sideroblasts, 4+ iron, erythroid hyperplasia</td>
<td>NP</td>
<td>Refractory sideroblastic anemia</td>
</tr>
<tr>
<td>H.H. (2 wk after transfusion)</td>
<td></td>
<td>12.0</td>
<td>0.5</td>
<td>29</td>
<td></td>
<td>sideroblasts, hyperplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H.L.</td>
<td>38/M</td>
<td>78.5</td>
<td>7.8</td>
<td>19.0</td>
<td>17</td>
<td>Extensive fibrosis and osteosclerosis, few normal cellular elements, no stainable iron,†</td>
<td>P</td>
<td>Myelofibrosis</td>
</tr>
<tr>
<td>C.D.</td>
<td>67/M</td>
<td>64.0</td>
<td>9.4</td>
<td>15.6</td>
<td>16</td>
<td>Erythroid hyperplasia, 2+ iron.</td>
<td>P</td>
<td>Myeloproliferative syndrome</td>
</tr>
<tr>
<td>E.I.</td>
<td>52/M</td>
<td>64.3</td>
<td>11.3</td>
<td>—</td>
<td>28</td>
<td>Myeloid hyperplasia, erythroid hypoplasia, no stainable iron,†</td>
<td>P</td>
<td>Acute myelomonocytic leukemia</td>
</tr>
<tr>
<td>V.P.</td>
<td>57/F</td>
<td>66.0</td>
<td>16.5</td>
<td>4.8</td>
<td>26</td>
<td>Erythroid hyperplasia, slight megaloblastosis, no stainable iron</td>
<td>P</td>
<td>Polycythemia vera</td>
</tr>
</tbody>
</table>

*P, palpable; NP, not palpable.
†Biopsy.
the appearance of $^{14}$CO in the breath reflects very recent heme-$^{14}$C catabolism and provides a useful tool for distinguishing sequential degradation of various heme pools.

Six patients were studied, including one normal subject. In an attempt to demonstrate a differential effect of erythropoietic activity on the early and late phase of the ELP, the size and contour of the early peak of a patient with sideroblastic anemia was assessed before and after transfusion. The other patients had various myeloproliferative disorders and were studied because information on the ELP in these conditions is not available in the literature.

SUBJECTS

Clinical data for the patients studied are outlined in Tables 1 and 2. They were apparently hematologically stable during the time of the investigations. The white cell and platelet counts for all patients were normal with two exceptions: C.D. had mild leukopenia (3790/cu mm) and a platelet count of 13,000/cu mm, while E.I.'s white count was 54,000/cu mm. There was no evidence of liver dysfunction in any patient except V.P. whose total bilirubin was 2 mg/100 ml and H.H. who had hemosiderosis by biopsy and a BSP retention of 11%. The only medications administered during the studies were allopurinol to H.L., phenylalanine mustard to V.P., and folic acid to C.D. H.H. underwent two series of studies 1 mo apart. Two weeks before the second investigation she received 4 U of packed red blood cells.

MATERIALS AND METHODS

Hematologic Methods

Multiple blood counts were performed for each subject by routine methods. The circulating red cell mass was measured by using chromium-51, and a chromium-51 half-life of the circulating red cells was determined. Total circulating hemoglobin was calculated as the product of the red cell mass and the mean corpuscular hemoglobin concentration. This value was converted to mmoles of circulating heme.

Measurements of the $V_{co}$

The daily endogenous production of carbon monoxide ($V_{co}$) was measured by the gas phase analysis method described by Logue et al.

Quantitation of the Early CO

Each subject received an intravenous injection of 100 $\mu$Ci of sterile, pyrogen-free glycine-$^{14}$C (specific activity, 4.9 $\mu$Ci/umole, New England Nuclear Corp.), and at various intervals thereafter samples of exhaled CO and peripheral blood were obtained.

Breath samples were collected in the rebreathing system used in the $V_{co}$ measurements. Each subject remained in the apparatus for 15–30 min while his exhaled CO accumulated. The gas in the rebreathing system was then flushed out with 100% oxygen into a large Douglas bag.

<table>
<thead>
<tr>
<th>Table 2. Clinical Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>H.L. (myelofibrosis)</td>
</tr>
<tr>
<td>C.D. (myeloproliferative syndrome)</td>
</tr>
<tr>
<td>E.I. (Myelomonocytic leukemia)</td>
</tr>
<tr>
<td>V.P. (polycythemia vera)</td>
</tr>
<tr>
<td>Normal values</td>
</tr>
<tr>
<td>Serum Fe</td>
</tr>
</tbody>
</table>
By keeping the O₂ concentration in the apparatus at greater than 90% during equilibration, it was possible to collect 5%-20% of the total body CO at each sitting. The concentration of CO in each breath sample was measured with an infrared analyzer (Beckman Instrument Associates). The volume of each sample was determined with a calibrated wet test meter (Precision Scientific Co.).

Since each breath collection contained a mixture of endogenous and exogenous CO, the CO content of each sample was multiplied by a fraction representing the endogenous portion only. This fraction was calculated from pulmonary parameters applied in the formulas of Coburn et al. for endogenous and exogenous carboxyhemoglobin saturation. For C.D., V.P., and E.I., pulmonary data were not available, and values for alveolar ventilation (6 liter/min) and CO-diffusing capacity (25 ml/min/mm Hg) were assumed. The pulmonary status of these patients was felt to be normal by clinical assessment and chest x-ray, except that E.I. had a small pleural effusion.

Daily breath samples were assayed for ¹⁴CO by techniques adapted from those of Landaw and Winchell. Each sample was drawn through a series of reagents including a CO₂ absorbent (500 g barium hydroxide lime, National Cylinder Gas), a water absorbent (150 g anhydrous CaSO₄, Fisher Scientific Co.), and an oxidizing catalyst (40 g, 60% MnO₂ and 40% CuO₂, Mine Safety Appliances Co.). The oxidized ¹⁴CO was collected and assayed by liquid scintillation counting according to methods described by Jeffay and Alvarez. The entire system was kept at room temperature. Flow rates were 400-500 ml/min. The efficiency and reproducibility of the assay were determined by measuring the recoverability of known amounts of ¹⁴CO (specific activity, 9.8 MCi/imole, New England Nuclear Corp.) from the apparatus. The efficiency of the system, which was included in the later calculations of the early peaks, was 0.47 ± 0.04 (SD). As indicated by the small standard deviation, the results were satisfactorily reproducible.

The specific activity (dpm/µmole endogenous CO) of each breath sample was plotted against time, and the average specific activity for each day multiplied by the \( V_0 \) to give the total radioactivity (expressed as dpms) exhaled as ¹⁴CO per day. The duration of the early CO peak was determined by extrapolating the experimental curve to 0. The possible error introduced by this extrapolation is discussed below.

**Heme Techniques, the Late CO Peak**

Hemin was isolated from 10-ml blood samples by the method of Labbe and Nishida and was recrystallized to constant specific activity. After being quantitated spectrophotometrically, each sample was combusted and the resultant ¹⁴CO₂ counted as described by Nathan et al. For the amounts of hemin used, the combustion efficiency was 0.94. This was determined by combusting known amounts of glycine-2-¹⁴C with various amounts of hemin. Sources of error in the assay system included the spectrophotometric measurements, radioactivity counting, and error related to the handling of samples. However, the reproducibility of the assay was good (SD less than 5% of mean).

The specific activity of each sample of circulating heme was plotted against time. The plateau of the derived curve was taken as the maximum specific activity resulting from effective erythropoiesis involving the available glycine-2-¹⁴C. This value was multiplied by the total quantity of circulating heme to give the total radioactivity appearing in the peripheral heme. For comparison with the ¹⁴CO production, this total radioactivity (dpms) was divided by eight, since each heme molecule contains eight possible ¹⁴C atoms, while CO contains only one. This value for circulating heme-¹⁴C was assumed to represent the ¹⁴CO which would arise as the late-labeled ¹⁴CO from the eventual senescence of the labeled cohort of red cells. Possible errors introduced by this assumption are discussed later.

**RESULTS**

The early ¹⁴CO peaks of the normal and myeloproliferative subjects are shown in Fig. 1, and the effect of transfusion on the ELP of H.H. is presented in Figure 2. The maximum rate of ¹⁴CO production by the abnormal subjects occurred 1-3 days later than that of the normal (J.H.). As Table 3 indicates, the size of the ELP in all of the patients was over twofold greater than that
observed for the subject with no hematologic disease. Furthermore, when the sum of the early and late ("potential") $^{14}$CO was calculated, the ELP was found to represent only 24% of this total in the normal subject, whereas it was at least twice that in all the abnormal patients except V.P. For V.P. the fraction ELP/(ELP + LLP) was equal to that for the normal, although the actual size of her ELP was over twice as large as that of the normal subject.

Two weeks after transfusion, H.H. received a second injection of glycine-$^{2,14}$C, which revealed that her early $^{14}$CO production was then one-quarter of its previous size. All parts of the early peak were affected, although the most dramatic change occurred on days 2–4.

For 4 days prior to her second glycine-$^{2,14}$C injection, the specific activity of H.H.'s peripheral heme was measured and found to be rising at a rate of 2 dpm/μmole/day. This rise was attributed to the senescence of the unlabeled transfused blood which the patient had received 2 wk earlier. Following the second glycine injection, the patient's heme specific activity was monitored for an additional 10 days and was observed to rise at the same rate. The failure of
Table 3. Early and Late Peak Data

<table>
<thead>
<tr>
<th>Subject</th>
<th>( V_{m} ) (( \mu \text{ mole/day} ))</th>
<th>Endogenous CO fraction*</th>
<th>( ^{51} \text{Cr} ) Red Cell Mass (ml)</th>
<th>Maximum Heme Specific Activity (dpm ( \times 10^{3} )/( \mu \text{ mole} ))</th>
<th>ELP (dpm ( \times 10^{3} ))</th>
<th>LLP (dpm ( \times 10^{3} ))</th>
<th>ELP ELP + LLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.H.</td>
<td>500</td>
<td>0.63</td>
<td>1367</td>
<td>87</td>
<td>89</td>
<td>287</td>
<td>0.24</td>
</tr>
<tr>
<td>H.H.</td>
<td>(Before transfusion)</td>
<td>580</td>
<td>0.75</td>
<td>720</td>
<td>112</td>
<td>438</td>
<td>175</td>
</tr>
<tr>
<td>H.H.</td>
<td>(After transfusion)</td>
<td>480</td>
<td>0.69</td>
<td>1024</td>
<td>0</td>
<td>111</td>
<td>0</td>
</tr>
<tr>
<td>H.L.</td>
<td>2190</td>
<td>0.87</td>
<td>2022</td>
<td>150</td>
<td>753</td>
<td>735</td>
<td>0.51</td>
</tr>
<tr>
<td>C.D.</td>
<td>1770</td>
<td>0.94</td>
<td>1089</td>
<td>260</td>
<td>1079</td>
<td>722</td>
<td>0.60</td>
</tr>
<tr>
<td>E.I.</td>
<td>1600</td>
<td>0.84</td>
<td>1840</td>
<td>71</td>
<td>437</td>
<td>315</td>
<td>0.58</td>
</tr>
<tr>
<td>V.P.</td>
<td>1180</td>
<td>0.87</td>
<td>2541</td>
<td>123</td>
<td>235</td>
<td>698</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*From the formulas of Coburn et al.\textsuperscript{13}
the slope of the heme specific activity curve to increase after the second injection of isotope suggested that the transfusion had completely suppressed effective erythropoiesis. Therefore, this patient's late-peak $^{14}$CO at that time was calculated as 0. However, the peripheral reticulocyte count of 0.5% (range, 0.1%–0.8%) at the same time indicated that at least some effective red cell production was still present. It was postulated that the discrepancy may have been related to the failure to assay heme specific activity for the first 2 days after the second glycine injection, a time during which a rise in heme specific activity could have been transient because of hemolysis of labeled reticulocytes. Another possible explanation is that, although a single straight line could be easily fitted to the values for the pre- and postinjection specific activities ($R = 0.91$), a longer period of observation might have demonstrated a slight change in slope.

**DISCUSSION**

**Size of the ELP**

The studies reported here demonstrate increases in the early $^{14}$CO excretion of the patients with erythropoietic disorders as compared with the early peak of the normal volunteer. The phenomenon of an enlarged early peak has previously been described for sideroblastic anemia,$^4$ although it has not been reported for myeloproliferative diseases.

In our normal subject (J.H.), 24% of the total $^{14}$CO production (i.e., the ELP plus the potential $^{14}$CO in the circulating heme-$^{14}$C) was observed to arise in the early peak of excretion. Figures in the range of 10%–20% have previously been reported for this fraction in normal subjects.$^{1,2,4}$ The larger value which we observed may have resulted from the technique used to measure the total CO production ($V_0$). This is discussed below.

In all of the hematologically abnormal subjects except V.P., the fraction ELP/(ELP + LLP) was much greater than in the normal volunteer (Table 3). Since the ELP of these patients apparently arose predominantly from erythropoietic activity (see below), it appears that increased erythroid heme degradation was playing a prominent role in the disease process of these individuals. The presence of such a phenomenon in sideroblastic anemia and myelosclerosis has also been suggested by ferrokinetic studies.$^{22,23}$

For the patient with polycythemia vera (V.P.), however, the fraction ELP/(ELP + LLP) was equal to that of the normal subject. This means that this patient's ELP had risen in proportion to her total red cell production, i.e., as a by-product of accelerated erythropoiesis without abnormal heme degradation. This interpretation is supported by ferrokinetic studies of polycythemia vera$^{22,24}$ which also failed to find evidence for "ineffective" erythropoietic activity until late in the course of the disease.

The effect of folate or iron deficiency on the ELPs of several of our patients should be considered. Because of the elevated early peak observed with the megaloblastic anemia of vitamin $B_12$ deficiency,$^5$ it is reasonable to assume that the folate deficiency present in H.L. may have contributed to his exaggerated ELP. However, the lack of megaloblastic changes in this patient's bone marrow suggested that folate deficiency was at least not a major influence on his eryth-
ropoiesis and hence could not have accounted for more than a minor fraction of his enormous early peak of $^{14}$CO.

Bone marrow iron stores were absent in H.L., V.P., and E.I., although the serum iron level was low only in V.P., and the serum iron-binding capacity was not elevated in any of the three (Tables 1 and 2). Therefore, only minor degrees of iron deficiency could have existed in any of these patients. Nevertheless, various studies have suggested that iron deficiency does lead to ineffective red cell production$^{25,26}$ and hence might be expected to elevate the early peak of heme catabolite excretion. This has in fact been demonstrated in rats,$^{27}$ but the increases in ELP were only three times normal in response to severe iron deficiency. Therefore, it is unlikely that the amounts of iron deficiency in H.L. or E.I. could account for more than a small portion of their huge early peaks. From the rat studies it would appear that V.P.'s early peak could be explained by iron deficiency. However, as noted above, her ELP was elevated in proportion to her LLP and was more likely a by-product of effective red cell production.

Although earlier studies have demonstrated a direct correlation between the erythroid cellularity of the bone marrow and the size of the ELP,$^{4,8}$9 only three of our five patients (H.H., C.D., V.P.) had marrow erythroid hyperplasia, and the other two (H.L. and E.I.) had hypoplasia. This discrepancy can perhaps be explained by sampling error in the bone marrow specimens, or more likely by the presence of extramedullary hematopoiesis in the enlarged spleens of these patients.

In E.I. the myeloid cell line was hyperplastic. We cannot rule out the possibility, therefore, that part of his ELP was related to the turnover of white cell heme proteins. It seems unlikely, however, that this process could have contributed the majority of his early $^{14}$CO, since the peak of his $^{14}$CO curve fell on days 2 and 3 when the major erythroid contribution to the early catabolite excretion is felt to occur$^{5,10}$ (see below).

Sources of Error

The measurement of the early-labeled $^{14}$CO production of our patients is subject to possible error from several sources:

(1) The size of the ELP, as we determined it, is critically dependent upon accurate measurements of the $V_{co}$, which were obtained by gas phase analysis in a rebreathing system. This technique indicates CO production rates about 40% higher than those measured by other methods,$^{28}$ although the reason for this discrepancy is unclear. Nevertheless, if the ELPs of our patients are calculated using an estimate of the $V_{co}$ which is 40% less than those we measured, great differences still exist between the normal and abnormal subjects. Overestimation of the $V_{co}$ would also create overestimation in the fraction ELP/(ELP + LLP).

(2) The fraction of the CO in the collected breath samples which arose from endogenous as opposed to environmental sources was calculated on the basis of the alveolar ventilation, carbon monoxide diffusion capacity, ambient carbon monoxide partial pressure, the $V_{co}$, and the assumptions discussed by Coburn et al.$^{15}$ The necessary pulmonary parameters were measured for all subjects
except C.D., V.P., and E.I., for whom the CO diffusion capacity and alveolar ventilation were assumed as 25 ml/min/mm Hg and 7 liters/min, respectively. The $V_{\infty}$ in these patients was high enough so that the effect of errors in these assumptions was minimized. A 40% error in the CO diffusion capacity combined with a 30% error in the alveolar ventilation would produce a maximum error of only 6% in the calculated endogenous CO fraction. Hence, an error introduced by these assumptions would not alter the final data significantly.

(3) Extrapolation of the late portion of several of the early peaks may have caused underestimation of their size, since it was assumed that the general shape of the ELP was symmetrical. However, a tendency for the late portion of the peak to slope more gently, as observed in H.H., may well have been present in some of the other subjects too.

Estimation of the size of the late peak (LLP) assumed that the CO production from the degradation of circulating hemoglobin is stoichiometric. This assumption has been confirmed in experimental animals. However, the comparison of early $^{14}\text{CO}$ excretion with potential $^{14}\text{CO}$ represented in the intravascular heme-$^{14}\text{C}$ is not valid if our estimates of the ELP were influenced by the possible technical error in $V_{\infty}$ measurements discussed above. Such a comparison would introduce a systematic error into the calculation ELP/(ELP + LLP).

The quantitation of total intravascular heme-$^{14}\text{C}$ is dependent upon the assumption that the heme-$^{14}\text{C}$ pool which we measured did in fact represent the accumulation of all the heme-$^{14}\text{C}$ delivered to the circulation and had not been reduced by peripheral hemolysis. The validity of this assumption is supported for the majority of our patients by the fact that their maximum level of circulating heme-$^{14}\text{C}$ remained stable for at least 2 wk. However, such a plateau was never prolonged in H.L. and C.D., the two patients whose chromium-51 survival half-times were substantially shortened. Therefore, measurement of the LLP of these subjects required an estimate of the size of the intravascular heme-$^{14}\text{C}$ pool. The amount of error which this estimate introduced into the calculation ELP/(ELP + LLP) for these patients is difficult to judge. Nevertheless, it is unlikely that such an error could explain the size of this fraction in these patients, since it represents greater than a twofold increase over that for the normal subject.

**Contour of the ELP. Effect of Transfusion**

The peak of the early $^{14}\text{CO}$ curve of the hematologically abnormal subjects consistently appeared later than that of the normal subject. Since in experimental animals it is the late phase of the ELP which is apparently related to erythropoiesis, the contour of the ELP in our abnormal patients indicates that the increased size of their peaks largely resulted from erythropoietic dysfunction. The effect of transfusion on the early $^{14}\text{CO}$ production of H.H. supports this hypothesis by demonstrating the sensitivity of the later part of the ELP to erythropoietic suppression.

Sampling the $^{14}\text{CO}$ excretion of H.H. during the first few hours after her injections of glycine-2-$^{14}\text{C}$, however, revealed that the earliest interval of her early peak was also damped by transfusion, although to a lesser degree than the
later phase. Other workers have shown that the earliest phase of the ELP does not vary with the degree of erythroid activity\textsuperscript{5,10} although this phase has been observed to rise following phlebotomy or phenylhydrazine-induced anemia in rats.\textsuperscript{5} Since in the post-transfusion study of H.H., breath samples were not taken until 2 hr after the glycine injection, it is possible that a brief unsuppressed phase of \textsuperscript{14}CO excretion was overlooked. Nevertheless, at 2 hr and again at 4 hr, a several-fold reduction in \textsuperscript{14}CO excretion was demonstrated in H.H. after transfusion (Fig. 2). This study, therefore, would suggest that, at least under conditions of erythropoietic stress, early \textsuperscript{14}CO related to erythroid heme catabolism adds to the earliest phase of the early peak. However, our observations do not indicate whether this “earlier” erythroid component in the ELP merely represents the leading edge of the major erythroid phase to follow, or whether it is the product of a separate, rapid catabolic event in red cell production. The existence of the latter has been previously postulated,\textsuperscript{31} but proof of either hypothesis awaits further investigation.

REFERENCES

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MK 3d Horne, WF Rosse, EG Flickinger and HA Saltzman