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Research Article

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Effect of Erythrocyte Destruction on Carbon Monoxide Production in Man *

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Recent studies performed in our laboratory (1) have confirmed the finding of Sjöstrand (2, 3) that carbon monoxide is produced in normal man. In 10 normal subjects the average rate of production of carbon monoxide was found to be 0.42 (SE of mean ± 0.02) ml per hour. The present study is an attempt to clarify the processes that result in the production of this gas in the body.

A number of studies have been performed that suggest that CO may be an *in vivo* catabolic by-product of hemoglobin. Sjöstrand and associates have demonstrated that the blood carboxyhemoglobin concentration [COHb] becomes elevated in man after intramuscular or intravenous injection of blood or hemoglobin (3) and intravenous injection of damaged erythrocytes or nicotinic acid (4). Sjöstrand (3) and later Engstedt (5) and Oski and Altman (6) noted that the blood [COHb] level is elevated in patients with hemolysis. Chemical studies by Sjöstrand (7, 8) and Ludwig, Blakemore, and Drabkin (9) have demonstrated that treatment of hemoglobin (7, 8) and pyridine hemochromogen (9) with hydrogen peroxide and ascorbic acid results in CO formation. In the latter study the bridge and adjacent carbon atoms of heme were labeled with C^{14}, and the CO formed was found to be radioactive, indicating that CO probably arose from a methene bridge carbon atom.

There remains, however, some uncertainty concerning the relationship of hemoglobin catabolism to endogenous carbon monoxide production *in vivo*. The elevated venous blood [COHb] found in the above *in vivo* studies may not be a result of an increased rate of endogenous CO production, since this variable is also markedly influenced by the concentration of CO in inspired air, the affinity of hemoglobin for CO, and the rate of excretion of CO from the body. Doubt has arisen concerning whether the chemical studies of Sjöstrand and Ludwig and associates are applicable to *in vivo* hemoglobin catabolism, especially since recent studies of Petryka, Nicholson, and Gray (10) have shown that coupled oxidation of heme is a different reaction than occurs physiologically during heme catabolism in that the porphyrin ring is apparently randomly opened at any of the four methene bridge carbon atoms rather than at the alpha position as occurs *in vivo* (11). Because of these uncertainties, our method for estimating *in vivo* CO production quantitatively was applied to this problem. This paper contains data on measurements of the rate of endogenous carbon monoxide production following the intravenous injection of damaged erythrocytes in man.

Methods

Endogenous CO production was measured by the "rebreathing method" described in greater detail elsewhere (1). The principle of this method is that CO excretion from the body, which occurs via the lungs, is inhibited by having the subject rebreathe in a closed system. Endogenous CO production is then calculated from the rate of increase in venous blood [COHb] during rebreathing and the effective blood volume, both of which are measured in every study. With this technique the production of CO per hour, calculated from two blood samples, drawn at the beginning and end of the period, could be estimated in each hour with an error of less than ± 0.2 ml. Greater precision was ob-

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tained during experiments in which the rate of CO production was measured by drawing and analyzing multiple blood samples. Here the error in measurement of the rate of CO production was less than ± 0.1 ml per hour.

The hemoglobin concentrations of the blood samples were measured as cyanmethemoglobin in a spectrophotometer (12). Plasma hemoglobin concentration was measured by the method of McCall (13). Base-line values by this method in our laboratory are 5 to 10 mg per 100 ml. Serum bilirubin was measured by the method of Malloy and Evelyn (14).

Human erythrocytes were "damaged" in the manner described by Jacob and Jandl (15). Fresh venous blood was drawn into a sterile bottle containing acid-citrate-dextrose (ACD) anticoagulant. The erythrocytes were collected by centrifugation and then were washed three times with cold physiological saline, and the final hematocrit was adjusted to 50%. The erythrocyte suspension was mixed with an equal volume of a solution of 32 mM *N*-ethylmaleimide (NEM) in 0.15 M NaCl giving 64 μmoles of NEM per ml of erythrocytes, and incubated at 37° C for 1 hour. After incubation the erythrocytes were again collected by centrifugation, washed once with 0.15 M NaCl, resuspended, and then reinjected intravenously into the donor. In two experiments the cells treated with NEM were labeled with radiochromate (16). Venous blood samples were taken periodically after reinjection of these cells, and the erythrocyte Cr^{51} activity was estimated in a well scintillation counter.

After preparation of the "damaged" erythrocytes, the subject initiated breathing in the closed circuit. The O_2 tension in the system was maintained at approximately 150 mm Hg by a demand valve, and CO_2 was absorbed. Fifteen minutes after the start of rebreathing a venous catheter was inserted into an antecubital vein, and the first blood sample was drawn anaerobically with a heparinized syringe. A small amount of mercury was added to the sample to facilitate complete mixing. Subsequent blood samples were taken hourly throughout the experiment and analyzed for [COHb], plasma bilirubin, plasma hemoglobin, and, in some experiments, erythrocyte radiochromate content.

Five experiments were performed with the subjects described in Table I. Four of the subjects were young male students who had normal peripheral blood hemoglobin concentrations, differential, leukocyte counts, and reticulocyte counts. The fifth subject was a 46-year-old female who was hospitalized with a pyridoxine responsive anemia. All of the subjects were either nonsmokers or abstained from smoking for 24 hours before the experiment. The "damaged" erythrocytes were labeled with radiochromate in the experiments on CB (Table I) and BH, a 54-year-old white male hospitalized with an uncomplicated duodenal ulcer, on whom the CO measurements were not performed.

In three of the experiments (RE, TP, and PK) the control rate of CO production was measured for 3 hours immediately preceding the reinjection of the "damaged" erythrocytes. In two experiments (BD and CB) the treated cells were injected at the start of the experiment. In all experiments, hourly CO production was measured for a total of 6 to 7 hours.

Because of the possibility that small amounts of unreacted NEM were injected with the erythrocytes, animal experiments were performed in which NEM in amounts equivalent to those used in the human experiments was injected intravenously into dogs, weighing 13 to 17 kg, anesthetized with pentobarbital. CO production rates were determined before and after giving the drug.

Results

After intravenous injection of the "damaged" erythrocytes CO was produced in greater amounts in all five of the experiments as compared to the control CO production (Table I). The changes in blood [COHb] and the corresponding hourly CO production from two of the five experiments are presented in Figures 1 and 2. The rate of increase in [COHb] in the 2 to 4 hours following injection of the treated cells was significantly greater in every experiment, being as much as 0.25% per hour, whereas the control rate averaged 0.05% per hour. The control rate CO production in the first three experiments (RE, TP, and PK) was 0.28, 0.43, and 0.46 ml per hour. It was necessary to use assumed values for the control CO production in the experiments on BD and CB, as will be discussed later. "Extra" CO was

TABLE I

Comparison of the amount of injected hemoglobin in the "damaged" erythrocytes and the "extra" CO produced

Subject	Age	Sex	Height	Weight	Control CO production	Hemoglobin injected		"Extra" CO		"Extra" CO / Heme injected
	yrs		*inches*	*lbs*	*ml/hr*	*g*	*μmoles heme*	*ml*	*μmoles*	*μmole/μmole*
RE	22	M	66	142	0.28	3.04	179.0	3.45	154	0.86
TP	29	M	69	170	0.43	2.92	172.0	3.48	155	0.90
PK	23	M	67	132	0.46	5.28	312.0	6.10	272	0.87
BD	22	M	68	170		3.37	198.5	5.90	263	1.32
CB	46	F	63	135		1.54	91.0	1.89	83.5	0.92

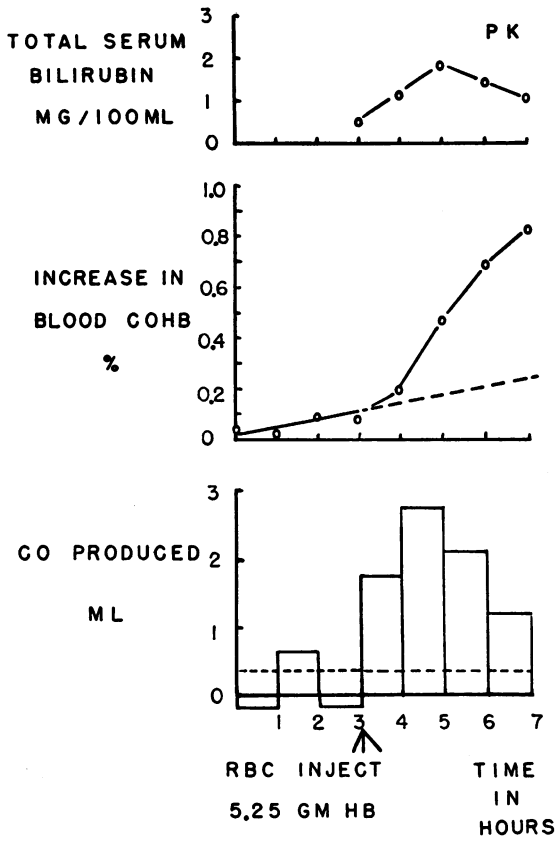

FIG. 1. EFFECT OF ERYTHROCYTE AND HEMOGLOBIN DESTRUCTION ON SERUM BILIRUBIN, RATE OF INCREASE IN BLOOD CARBOXYHEMOGLOBIN, AND HOURLY CARBON MONOXIDE PRODUCTION. The base-line carbon monoxide production was measured during the first 3 hours of the experiment followed by intravenous injection of erythrocytes prepared by incubation with 64 μmoles *N*-ethylmaleimide per ml erythrocytes for 1 hour at 37° C. The dashed line in the middle and bottom graph represents the average control rate of CO production.

calculated by subtracting the control from the total CO production, as will be discussed later. These values are listed in Table I. Up to 6.30 ml more CO was formed in the 2 to 4 hours following injection of the cells than would have been formed in an equivalent time at the control rate.

The injected erythrocytes were rapidly removed from the vascular compartment, as expected from the work of Jacob and Jandl (15). In the two experiments employing erythrocytes labeled with Cr^{51}, one-half of the radioactivity disappeared from the blood in seven (CB) and eleven (BH) minutes, and an average of less than 3% of the injected cells remained in the blood 1 hour after injection. The labeled cells did not return to the vascular compartment after they had been trapped at the site of sequestration.

Serum bilirubin levels increased significantly after injection of the erythrocytes (Figures 1 and 2). The increase in serum bilirubin levels appeared to parallel CO production.

The plasma hemoglobin increased slightly in three experiments where it was measured. Values up to 5 mg per 100 ml above the control levels were found in the first hour following injection of the cells, and these fell to control levels by the end of the third hour after injection. The quantity of hemoglobin appearing in the plasma was only a small fraction of the total hemoglobin administered in the cells and was not considered significant. Further, we have recently found that elevated serum bilirubin concentrations cause

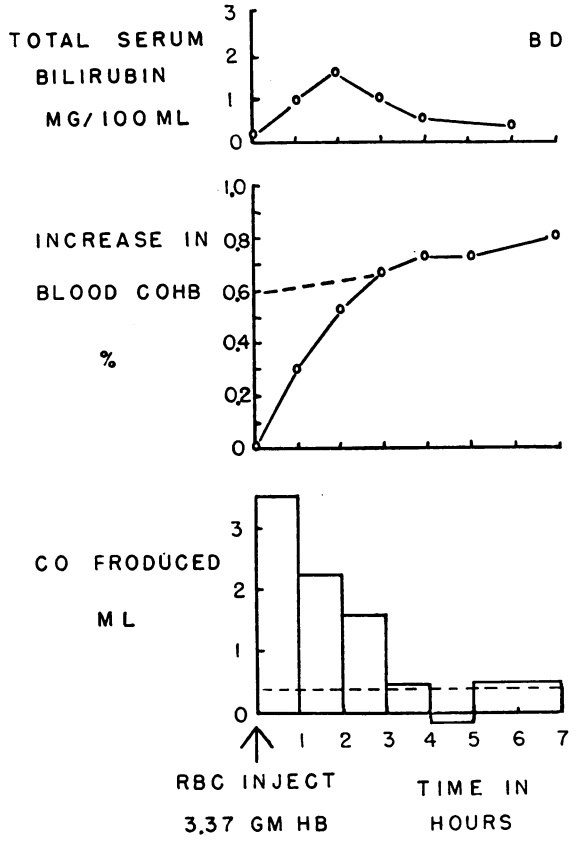

FIG. 2. EFFECT OF ERYTHROCYTE AND HEMOGLOBIN DESTRUCTION ON SERUM BILIRUBIN, RATE OF INCREASE IN BLOOD CARBOXYHEMOGLOBIN, AND HOURLY CARBON MONOXIDE PRODUCTION. The treated cells were injected at the beginning of the experiment.

falsely high values for plasma hemoglobin by the method used, and the real increase in plasma hemoglobin was probably even less than that recorded here.

Control studies were performed on two dogs in which the rate of CO production was measured before and after the intravenous injection of NEM in saline, in quantities comparable to those used in the human studies. The base-line CO production rate in these dogs was one-half to one-third that of the normal human, and was not changed by administration of NEM.

Discussion

The studies reported here demonstrate that CO is produced in large quantities after injection of erythrocytes treated so as to lead to their rapid removal from the circulation. The errors involved in estimating CO production have been presented in the Methods section and discussed in a previous publication (1). Measurement of total CO produced should be accurate to better than ± 10%.

It is assumed that the "extra" CO found was formed during hemoglobin catabolism via normal pathways. Although it seems unlikely, it is possible that injection of erythrocytes treated with NEM may stimulate CO production by some process other than hemoglobin degradation. The control experiments in dogs demonstrated that NEM itself is inert in this regard. The assumption that CO was produced as a result of hemoglobin degradation is supported by the demonstration that the serum bilirubin levels rose an average of 1.2 mg per 100 ml and appeared to parallel the CO production. The two studies employing cells labeled with Cr^{51} demonstrated that the administered hemoglobin was rapidly and nearly completely removed from the circulation and did not reappear for the duration of the experiment. Although we have assumed that the hemoglobin contained in the sequestered cells was catabolized, it was not possible to measure directly the amount of hemoglobin degraded in these experiments by an independent method.

In these studies it was important that the administered erythrocytes be sequestered promptly, so that complete hemoglobin degradation might be expected to occur during the time the subjects could be maintained in the rebreathing apparatus. NEM was chosen as a convenient agent to alter the erythrocytes, so that they would be rapidly sequestered *in vivo* but not hemolyzed before injection (15).

In the experiments on subjects RE, TP, and PK the control CO production was measured before injection of the treated cells, and the values found were similar to those previously reported for normal male subjects (1). In each of these subjects the rate of CO production in the final hour of the study was still greater than the control value, and it was therefore necessary to alter the experimental design to determine precisely the time relationship of production of CO after injection and sequestration of the treated cells. For this purpose studies were performed on two subjects, BD and CB, in which the treated cells were injected at the beginning of the experiment and estimation of CO production was continued until a constant rate was attained. In the experiment on BD, treated cells containing 3.37 g of hemoglobin were injected (Figure 2). A total of 7.3 ml of CO was produced in the first 3 hours, after which the production appeared to remain constant at a rate of 0.23 ml per hour. In the experiment on CB, 1.54 g of hemoglobin was injected. In the first 2 hours 2.69 ml of CO was produced, whereas 0.04 ml was produced in the second and third 2-hour periods. It appears from these experiments that nearly all the "extra" CO is produced within 3 hours after injection of treated cells containing up to 3.3 g of hemoglobin, and we are justified in assuming that all, or nearly all, of the "extra" CO was measured in the first two experiments, subjects RE and TP. Subject PK received 5.28 g of hemoglobin and still produced a slightly elevated quantity of CO in the fourth hour after receiving the treated cells. By comparison with the other studies, apparently here too essentially all the "extra" CO was measured. Possibly CO production is delayed slightly after injection of larger quantities of hemoglobin. The average half-time of CO production following cell injection was 87 (SE of mean ± 3) minutes. This value is certainly a function of several variables, and determination of rate constants or definition of limiting reactions is impossible at present.

With these data we have calculated the ratio of "extra" CO produced to the quantity of heme

administered in the treated cells. In these calculations we have assumed that the base-line rate of production of CO remains constant throughout the experiment. The "extra" CO produced after injection of the treated erythrocytes is then the difference between the total and base-line CO production. The results are presented in Table I. "Extra" CO was calculated in subjects RE, TP, and PK with the base-line CO production actually measured at the beginning of the experiment. For subjects BD and CB we used the average normal rate of CO production, 0.42 ml per hour (1). This was necessary because in subject CB the rebreathing was unavoidably interrupted briefly twice during the last hour of the base-line measurement, and thus the values obtained are of uncertain significance. In the experiment on BD the base-line rate of CO production was determined during the latter part of the experiment and was significantly lower than the normal rate previously reported (1). We suggest that the large amount of CO produced and added to the blood stores during the first 2 to 3 hours of the experiment had not reached complete equilibrium with the extravascular stores; therefore, there was a small loss of CO from the blood to the extravascular stores during measurement of the base-line rate, leading to underestimation of this value. In this experiment 5.9 ml of CO was produced during the first 2 hours of the experiment, and if only 5% of this quantity moved from the blood to the extravascular space in the period during which the base line was measured, this would have caused an underestimation of the rate of CO production of 0.09 ml per hour, or about 20% of the normal rate.

In Figure 3 the "extra" CO produced in micromoles is plotted against the micromoles of heme injected. There is a significant correlation between these variables ($r = 0.93$), and the average molar ratio of "extra" CO to heme is 0.97 (SE of mean $\pm$ 0.06). It appears from the data presented that CO is produced in man as a result of hemoglobin degradation, and the quantity produced is approximately 1 mole of CO per mole of heme catabolized. The precise source of CO is as yet undefined; however, these findings are consistent with its origin from the α methene carbon atom of heme. The yield of CO from hemoglobin is higher than others have previously found for

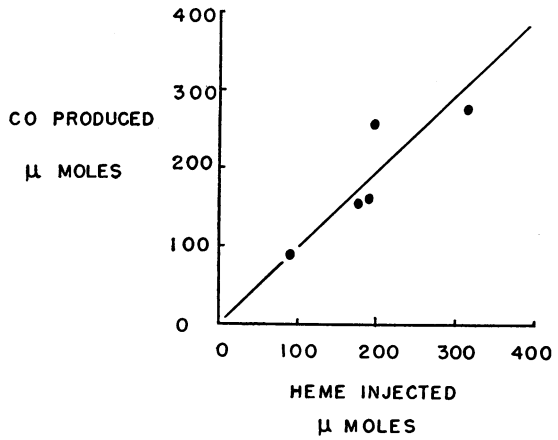

FIG. 3. COMPARISON OF THE AMOUNT OF INJECTED HEME AND THE "EXTRA" CARBON MONOXIDE PRODUCED IN FIVE EXPERIMENTS. Both of these variables are expressed in micromoles.

bile pigment production from hemoglobin, where yields of 50 to 80% have been reported (17–20). An explanation of this apparent discrepancy between the efficiency of CO production from heme and bile pigment production from heme may lie in the fact that CO is probably produced in the initial steps of hemoglobin catabolism (21, 22) and is metabolically inert (23), whereas the remaining tetrapyrrole moiety is subject to a variety of reactions in the process of production of the bile pigments.

On the basis of the present study it seems very probable that CO produced as an end product of hemoglobin catabolism forms a large percentage of the normal CO production. In the study previously reported (1) it was found that the average normal rate of CO production is 0.42 $\pm$ 0.02 ml per hour. The average total body hemoglobin in this group of subjects was 724 g. The average hourly rate of hemoglobin catabolism, assuming a mean erythrocyte survival time of 120 days, was 0.25 g per hour. This would result in a rate of carbon monoxide production, assuming a 0.97 M ratio, of 0.32 ml per hour from this source. Therefore, approximately 0.10 ml per hour (the normal rate of 0.42 ml per hour minus 0.32 ml per hour) or 23% of the total average rate of CO production must arise from sources other than circulating hemoglobin. In this regard London, West, Shemin, and Rittenberg (24) found that about 10 to 15% of normal urobilin production arose from sources other than circulating hemo-

globin. Possibly this portion of bile pigment production and approximately 0.10 ml per hour of CO may originate from the same or similar processes.

Summary

We have investigated the effect of an increased rate of hemoglobin destruction upon the rate of endogenous CO production in one female and four male subjects. The hemoglobin destruction rate was increased by reinjecting the subject's own erythrocytes which had been treated with *N*-ethylmaleimide. In all subjects the rate of CO production increased after the injection of the cells. The amount of this increase corresponded to the formation of an average of 0.97 ± 0.06 mole CO for each mole of heme destroyed. This study provides evidence that CO is an *in vivo* end product of heme catabolism.

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