ABSTRACT

The endogenous production of carbon monoxide and the flow of hemoglobin to and from plasma were measured in 11 anesthetized dogs after pumping blood through an extracorporeal circuit for short periods. Two different pumps were used. In all animals the increase in CO production was greater than could be explained by catabolism of hemoglobin lost from plasma, an average of 11.4 times greater with one pump and 2.49 times greater with the other pump. Evidence is presented that this discrepancy could not be explained by catabolism of heme other than that of hemoglobin, and we therefore concluded that rates of hemoglobin catabolism were much greater than indicated by plasma hemoglobin kinetics and that extravascular hemolysis is a major cause of erythrocyte destruction during mechanically induced hemolysis. Extravascular hemolysis apparently caused an average of 72.9% and 37.2% (with the two pumps) of the total quantity of erythrocytes destroyed during pumping and for 3 hours after pumping.

ADDITIONAL KEY WORDS

carbon monoxide production
erthrocyte destruction
extracorporeal circulation
100 ml water) were administered at a rate of approximately 100 ml/hour. To prevent loss of endogenously produced carbon monoxide, a closed rebreathing system with a mechanical ventilator was used, as previously described (1). A long polyethylene catheter was inserted into an external jugular vein for withdrawal of blood samples, and a catheter was inserted into the bladder. In several animals a small catheter was placed in a carotid or femoral artery during parts of the experiment so that arterial blood pressure could be monitored by a strain gauge and recorder and blood could be drawn for determination of blood gas tensions and, pH. The extracorporeal circuit was a simple closed system without an air-gas interface, consisting of either a finger pump (Sigmamotor, Model T6) with a latex rubber ventricle (5/8 inch i.d.) and 8 inch Tygon tubing (pump A), or a small roller pump (Masterflex Tubing Pump, Cole-Parmer) with a rubber ventricle (3/16 inch i.d.) and Tygon tubing (% inch i.d.) (pump B). The occlusiveness of pump B could not be adjusted. The total priming volume of pump A was 60 ml and that of pump B, 25 ml. The pumps were calibrated by in-vitro perfusion with normal saline. The flow rate decreased less than 5% against a pressure of 120 mm Hg. Veno-arterial perfusion was accomplished by inserting a stainless steel cannula into a carotid artery and a Silastic catheter through the jugular vein into the right atrium. All materials used were clean but not sterile. The preparation for these experiments is shown schematically in Figure 1.

In all of the experiments in which blood was pumped in the extracorporeal circuit, the following protocol was used. At the beginning of the experiment, 3 to 5 µc of 14 CO was injected into the rebreathing system, from which it was rapidly absorbed and mixed in the body CO stores. Serial measurements of blood carboxyhemoglobin percent saturation ([COHb]) and plasma hemoglobin concentration were then made over a 2-hour control period. Heparin, 30 units/kg, was given intravenously just before pumping. During and/or following pumping, blood samples were collected every 20 minutes and analyzed for blood 14CO radioactivity ([14CO]), [COHb] and plasma hemoglobin concentration. At the end of the experiment CO was given and the CO capacity determined (4).

In 11 experiments blood was pumped in the extracorporeal circuit for 15 to 135 minutes. Flow rates ranged from 77 to 540 ml/min with pump A and from 400 to 440 ml/min with pump B. After infusion of the pump contents, the cannulas were removed and the serial measurements described above were performed. In five of these experiments a tracer amount of 51Cr-labeled hemoglobin was injected intravenously at the beginning of the experiment. After 15 minutes a blood sample was collected and the plasma analyzed for 51Cr radioactivity, which was used in calculating the "plasma hemoglobin compartment," i.e., the hemoglobin dilution space. Serial measurements of plasma 51Cr radioactivity were made for the remainder of the experiment.

Two control experiments showed that the rate of CO production and plasma hemoglobin concentration were not significantly altered by arterial and venous cannulation or by heparin.

Nine additional experiments were performed to assess the possibility that function of the reticuloendothelial system was altered as a result of pumping blood in the extracorporeal system and that this alteration might have influenced the CO data.

1 Unless otherwise indicated, CO refers to carbon monoxide with a carbon neutron number of 12.

Animal preparation. The extracorporeal circuit was connected from right atrium to left carotid artery.

was determined every 10 minutes after injection, allowing calculation of sequestration of the labeled, damaged cells. Other measurements were made as described above.

2. In three experiments approximately 60 ml of blood was pumped in vitro in a gas-free closed circuit with pump A at a rate of 500 ml/min. After 15 to 30 minutes of pumping, the blood was removed from the circuit. In two experiments, it was injected intravenously into the donor animal and in the third it was centrifuged and the plasma (25 ml) was injected into the donor animal.

3. In three pump A experiments, after baseline measurements were made, blood was pumped in the extracorporeal circuit continuously for periods ranging from 165 to 180 minutes.

4. In one experiment we assessed the possibility that tissue hypoxia might occur in the reticuloendothelial system during or after perfusion and influence our measurements. With the animal breathing gas with a Po2 of 150 mm Hg, 14CO was administered into the rebreathing system and serial measurements of [COHb] were made over a 2-hour period. The Po2 of the inspired gas was then decreased to less than 60 mm Hg, and serial measurements of [COHb] and [14CO] were performed during the subsequent 2-hour period. Blood was not pumped in this experiment.

The standard method of determining the rate of carbon monoxide production (4) is based on the assumption that the partition between blood and extravascular CO stores is constant and that total body stores can therefore be computed from measurements of [COHb] and a dilution factor. This assumption has been supported by studies of awake human subjects (4, 6, 7) and anesthetized dogs (8) in which 14CO was administered and blood levels remained essentially constant for long periods after mixing (except for a very small constant rate of decrease due to metabolism of the isotope). However, in preliminary experiments in the present study, [COHb] sometimes decreased during and after pumping. Previous studies have shown that this phenomenon occurs during severe arterial hypoxemia (8, 9) and during shock (unpublished data) as a result of shifts of CO into muscle. Our explanation of this finding is that during tissue hypoxia the affinity of CO for myoglobin in skeletal muscle increases (9). We modified our method of measuring the rate of CO production by adding 14CO to the body stores to compensate for the change in partition of CO between vascular and extravascular CO stores.

The body 14CO radioactivity is assumed to remain constant throughout the experiment:

\[ X(t) = X(0), \]

where \( X(t) \) and \( X(0) \) are total body 14CO radioactivity at time \( t \) and time zero.

It is assumed that the specific activity of CO (SA) at any given time is the same everywhere in the body. SA is defined as follows:

\[ SA(t) = X'(t)/X(t), \]

where \( X(t) \) is the total quantity of CO in the body at time \( t \).

Rearrangement of the above equations yields

\[ X(t) = SA(t) \times X(t), \]

and similarly for time zero,

\[ X(0) = SA(0) \times X(0). \]

From equation 1

\[ X(t) = X(0) \times SA(0)/SA(t). \]

In our experiments we computed \( X(t) \) from blood specific activity measurements taken every 30 to 60 minutes using equation 5 and plotted \( X(t) \) vs. time. In this calculation, \( X(0) \) was determined from the initial [COHb] and the CO capacity measurement (4). From these data we could compute increases in the rate of CO production (Vco) by subtracting the baseline Vco from that determined following extracorporeal circulation. In anesthetized dogs, the baseline Vco remains constant within the error of measurement for up to 8 hours (8), and evidence has been obtained that it does not change after intravenous injection of hemoglobin solution or damaged erythrocytes (1). Since the partition of CO between blood and extravascular tissues did not change before pumping, we determined the baseline Vco from the increase in [COHb] alone (4). The error in determining baseline Vco was ±0.03 ml/hour; after pumping, the error was slightly greater due to the additional errors of the 14CO measurement. The assumption that the total body 14CO radioactivity remains constant appears justified on the basis of previous studies, which showed a very small rate of metabolism of CO (8). The rate of oxidation of 14CO to 14CO2 following pumping in one experiment was only 0.16% of the total body 14CO radioactivity. In addition, the previously mentioned studies
obtained during hypoxemia and shock (8) have shown that the shift of \( \text{CO} \) into muscle is reversible. The assumption that specific activity is equal in all of the body \( \text{CO} \) stores is tantamount to saying that mixing of \( \text{CO} \) in all of the stores is infinitely rapid. This is obviously not correct, but it has been shown that under normal conditions in anesthetized dogs, and when \( \text{CO} \) has shifted into muscle, mixing of \( \text{CO} \) is sufficiently rapid (8) that significant error in calculating \( V_{\text{CO}} \) could not have occurred in our experiments.

MEASUREMENT OF EFFLUX OF HEMOGLOBIN FROM PLASMA

We measured the plasma hemoglobin compartment by injecting a known quantity of \( \text{SI} \) labeled hemoglobin intravenously and determining the plasma hemoglobin radioactivity 15 minutes after injection. Rates of efflux of hemoglobin from plasma were then computed from this dilution term and rates of decrease of the plasma hemoglobin concentration during periods following cessation of pumping. The \( \text{SI} \) labeled hemoglobin was prepared by washing dog erythrocytes twice in cold saline and incubating them with \( \text{Na}_2\text{CrO}_4 \) at 37°C for 1 hour. The cells were then washed three times and lysed by freezing and thawing three times. The supernatant fluid was diluted in an isotonic sodium phosphate buffer, pH 7.4. This solution was dialyzed against the phosphate buffer at 2°C for 24 hours or until over 98% of the dialyzable radioactivity was removed. The resultant solution was kept frozen until use.

We evaluated the possibility of elution of the label from the hemoglobin in five animal experiments in which \( \text{SI} \) hemoglobin and nonradioactive hemoglobin (prepared by freezing and thawing) were injected into the vascular compartment simultaneously. Blood specimens were drawn 15 minutes after injection and every 30 minutes for the next 4 to 5 hours. The specific activity of the plasma hemoglobin decreased for approximately 2 hours after injection, suggesting that elution of the radioactive tag occurred during this period, but it remained constant thereafter. Measurements of plasma specific activity in four of these experiments are shown in Figure 2. Initial plasma hemoglobin levels ranged from 53 to 98 mg/100 ml, and the plasma hemoglobin concentration decreased at an average rate of 4.5 ± 1.2 mg/100 ml/hour. The plasma hemoglobin compartment in these similar experiments averaged 77 ml/kg. If there were any chemical dissimilarities between the labeled and unlabeled hemoglobin due to differences in preparation, they apparently did not significantly influence rates of loss of hemoglobin after 2 hours postinjection. These experiments suggest that the injected labeled hemoglobin could be used in computing rates of efflux of hemoglobin from plasma after 2 hours postinjection.

Calculation of efflux of hemoglobin from plasma using the plasma hemoglobin compartment and the rate of decrease of the plasma hemoglobin concentration could be inaccurate if hemoglobin was being added to the plasma while efflux was occurring. The injection of labeled hemoglobin allowed us to evaluate this possibility since the rate of efflux at any given time could be computed from the rate of change of specific activity of plasma hemoglobin and the total quantity of acellular hemoglobin in the extravascular space at that time. As noted above, the radioactive hemoglobin was always injected at least 2 hours before pumping so that we could be relatively certain that elution of the label was not occurring.

ANALYTICAL TECHNIQUES

Plasma hemoglobin concentrations were determined by the method of Crosby and Furth (10). Blood \( [\text{COHb}] \) was measured by an infrared method (11). Blood \( [\text{U CO}] \) was determined in an ionization chamber after extraction from 2 ml of blood (8). \( \text{U} \) nuclide disintegration was measured in a NaI well scintillation counter. Blood gas tensions and pH were determined with appropriate electrodes (12, 13).

Results

Baseline data are presented in Table 1. Baseline rates of \( \text{CO} \) production did not differ significantly from those obtained in a previous

![Figure 2](http://circres.ahajournals.org/)

**Figure 2**

Specific activity of plasma hemoglobin following simultaneous intravenous injections of hemoglobin labeled with \( \text{U} \) into anesthetized dogs. The specific activity of plasma hemoglobin decreased for approximately 2 hours after injection but following this remained constant. These data suggest that no elution of the radioactive tag occurred after 2 hours postinjection. Symbols identify different dogs.
MEASUREMENT OF MECHANICALLY INDUCED HEMOLYSIS

### Baseline Data

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<tr>
<th>Dog no.</th>
<th>wt (kg)</th>
<th>Plasma Hb concn (g/100 ml)</th>
<th>Baseline Vco (ml/hr)</th>
<th>Pump rate (ml/min)</th>
<th>Pump time (min)</th>
<th>Plasma Hb compartment (ml/kg)</th>
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### Pump A Experiments

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<th>Baseline Vco (ml/hr)</th>
<th>Pump rate (ml/min)</th>
<th>Pump time (min)</th>
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### Pump B Experiments

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<th>Baseline Vco (ml/hr)</th>
<th>Pump rate (ml/min)</th>
<th>Pump time (min)</th>
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<td>0.11</td>
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### Continuous Pump A Experiments

A decrease in blood $[^{14}CO]$ occurred in 9 of the 11 experiments in which short pumping periods were used and in all of the "continuous" pump experiments (Figs. 3 and 4). In general, larger decreases in $[^{14}CO]$ occurred in the continuous pump experiments and in those conducted at higher flow rates. This loss of $[^{14}CO]$ from the blood could not be attributed to arterial blood hypoxemia (8), since the arterial $P_{O_2}$ did not fall below 60 to 70 mm Hg during or after pumping; nor could it be attributed to hypotension, since systolic blood pressures ranged from 90 to 100 mm Hg during pumping (compared with values of 90 to 130 mm Hg before pumping).

The plasma hemoglobin concentration increased during pumping in all but one of the experiments. Maximal concentrations ranged from 8.3 to 55 mg/100 ml with pump A and from 55 to 79 mg/100 ml with pump B (Table 2). The urinary threshold apparently was not exceeded in any of the animals, as judged by visual examination of the urine. Total quantities of hemoglobin that entered the plasma ranged from 0.0 to 48.2 $\mu$mols heme with pump A and from 42.7 to 64.1 $\mu$mols heme.

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"Throughout this paper the ± values are standard errors of the means.

*Circulation Research, Vol. XXVI, March 1970*
Table 2

### Effect of Pumping on Plasma Hemoglobin Catabolism and CO Production

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>Plasma Hb concentration (mg/100 ml)</th>
<th>Rate of decrease (mg/100 ml/hr)</th>
<th>Highest Vco₂ (ml/hr)</th>
<th>Time (min)</th>
<th>Plasma Hb loss (gimoles/hour)</th>
<th>Excess CO produced (gimoles)</th>
<th>Contribution of plasma Hb catabolism to excess CO (%)</th>
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*Measured over a 1-hour period. †We assumed a plasma hemoglobin compartment of 77 ml/kg in calculating plasma hemoglobin loss.

with pump B (Table 3). After perfusion, the plasma hemoglobin concentrations decreased at rates ranging from 0 to 7.1 mg/100 ml/hour (Table 2). These rates of decrease were grossly similar to those found in the control experiments, in which hemoglobin solution was injected and pumping was not performed, and peak plasma hemoglobin concentrations were equivalent. These findings suggest that if alterations in function of the reticuloendothelial system occurred during pumping, they did not affect the variables that determine rates of catabolism of the hemoglobin-haptoglobin complex.

In the pump experiments in which ⁵¹Cr-labeled hemoglobin was injected, the plasma hemoglobin compartment averaged 77 ml/kg, and because this value was not critical for the purposes of our study we used it in calculating rates of efflux of hemoglobin from plasma in the other pump experiments also. As calculated from rates of decrease in plasma hemoglobin concentration and the plasma hemoglobin dilution factor, hemoglobin lost from the plasma at rates ranging from 10 to 83 mg/hour. In the experiments in which labeled hemoglobin was injected, the specific activity of the plasma hemoglobin did not vary during the first 3 to 4 hours after pumping; therefore, calculations made from measurements of the rate of decrease of this tracer gave values not significantly different from those calculated from rates of decrease of the plasma hemoglobin concentration.

Table 2 includes a comparison of CO production in excess of baseline production and the total quantity of hemoglobin lost from the plasma hemoglobin compartment during a 5- to 4-hour postperfusion period. In each of the pump experiments the quantity of CO...
MEASUREMENT OF MECHANICALLY INDUCED HEMOLYSIS

FIGURE 3

Data obtained in a typical pump experiment. Top: Measurements of plasma hemoglobin concentration and radioactivity and their ratio, with the specific activity of plasma hemoglobin given as counts per minute per milligram of hemoglobin. Tracer quantities of $^{14}$C-labeled hemoglobin were injected at arrow, and measurements were not taken for 2 hours because the tag apparently is eluted from hemoglobin for 2 hours following injection, as shown in Figure 2. Bottom: Plot of the increase in body CO stores before and after pumping. The broken line is an extrapolation of the baseline CO production. This figure illustrates the increase in rate of CO production after pumping that occurred in every experiment, the loss of hemoglobin from plasma, and the finding that the specific activity of plasma hemoglobin remained constant following pumping, indicating that there was no further influx of hemoglobin into plasma.

### TABLE I

<table>
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<th>Day no.</th>
<th>IH (mmol/l)</th>
<th>EH (mmol/l)</th>
<th>Time of EH (min)</th>
<th>IH/EH (%)</th>
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<td>0.57</td>
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<tr>
<td>SE</td>
<td>6.1</td>
<td>16.1</td>
<td>8.5</td>
<td>0.25</td>
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</tr>
</tbody>
</table>

**Pump B Experiments**

<table>
<thead>
<tr>
<th>Day no.</th>
<th>IH (mmol/l)</th>
<th>EH (mmol/l)</th>
<th>Time of EH (min)</th>
<th>IH/EH (%)</th>
<th>IH/EH</th>
<th>IH/EH</th>
</tr>
</thead>
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<tr>
<td>9</td>
<td>54.0</td>
<td>27.3</td>
<td>150</td>
<td>33.6</td>
<td>1.96</td>
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<td>10</td>
<td>66.1</td>
<td>4.7</td>
<td>180</td>
<td>6.8</td>
<td>13.64</td>
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<td>11</td>
<td>42.7</td>
<td>107.8</td>
<td>180</td>
<td>71.6</td>
<td>0.40</td>
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<tr>
<td>Mean</td>
<td>52.6</td>
<td>66.6</td>
<td>37.3</td>
<td>5.34</td>
<td>2.56</td>
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<tr>
<td>SE</td>
<td>6.2</td>
<td>31.3</td>
<td>18.8</td>
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</tbody>
</table>

IH = intravascular hemolysis; EH = extravascular hemolysis.

Data obtained in a constant-pumping experiment. This plot illustrates measurements of blood [UCO] radioactivity ([UCO]) and blood carboxyhemoglobin percent saturation ([COHb]), from which we calculated changes in the body CO stores. As in the other pump experiments, the rate of CO production increased with pumping of blood in the extracorporeal circuit. Broken line is an extrapolation of the baseline CO production.

Results obtained in the experiments designed to evaluate the effects of pumping blood in the extracorporeal circuit on the ability of the reticuloendothelial system to sequester erythrocytes and produce CO were as follows:

1. NEM-damaged erythrocytes (containing 5.45 g hemoglobin) injected at the end of the pumping procedure were removed from the circulating blood with a half-life of 15 minutes, and 98% were removed within 2 hours. The rate of CO production increased from a baseline value of 0.09 ml/hour to 1.7 ml/hour within 1 hour and remained constant for the duration of the experiment. In the other experiment NEM-damaged erythrocytes (containing 1.98 g hemoglobin) injected during perfusion were sequestered at a much slower rate (t1 39 minutes), but after cessation of pumping the rate of disappearance from circulating blood increased markedly (t1 8 minutes). As in the first experiment, more than 98% of the injected cells were ultimately removed from the circulating blood. The rate of CO production increased from a baseline value of 0.15 ml/hour to a maximum of 1.10 ml/hour. In these experiments the plasma hemoglobin concentrations at the end of the pumping procedure were 45 mg/100 ml and 58 mg/100 ml, respectively. In comparison with data obtained in similar, previously reported experiments (2), which can be regarded as control experiments since no extracorporeal circuit was used, it appears that sequestration of erythrocytes may be delayed during pumping of blood but occurs at a normal rate after perfusion, and that the maximal CO production after perfusion is also normal. Data from these experiments are shown in Figure 5.

2. Data obtained in the experiments in which we pumped blood in an in-vitro circuit and reinfused it into the donor animal were as follows. Within 2 hours after reinfusion Vco increased from a control value of 0.20 ml/hour to 1.8 ml/hour in one animal and from 0.18 ml/hour to 1.1 ml/hour in the other animal. In the experiment in which erythrocytes were removed from blood that had been pumped in vitro and the plasma reinfused, the baseline Vco was 0.42 ml/hour, and Vco did not change significantly in the 3 hours following reinfusion. In this experiment the plasma hemoglobin concentration of the blood in the in-vitro system reached 920 mg/100 ml and 175 mg of hemoglobin was infused into the circulation of the animal. About half of this
was removed from the animal's plasma during
the 3-hour measurement period; this would
have resulted in a Vco of only 0.04 ml/hour,
which is not detectable. These findings
suggest that the increase in Vco observed
after pumping in vivo did not result from
hemodynamic effects of pumping unrelated
to erythrocyte damage, or from alterations
of plasma or effects of plasma alteration on the
reticuloendothelial system or undamaged
erythrocytes.

3. In the continuous pump A experiments,
maximal rates of CO production averaged
0.50 ± 0.13 ml/hour, a value not significantly
different (P > 0.05) from that obtained in the
other pump A experiments, suggesting that
pumping per se did not markedly influence
the ability of the reticuloendothelial system to
produce CO.

4. In the experiment in which the effects of
severe hypoxia were studied, Vco did not
change significantly during the 3-hour period
when arterial P02 was maintained between 35
and 50 mm Hg, compared to the baseline Vco
(Fig. 6). If tissue hypoxia occurred during
pumping, it apparently did not significantly
affect the ability of the reticuloendothelial
system to produce CO at the baseline rate.

Discussion
A principal finding in these studies was that
after pumping of blood in the extracorporeal
circuit the quantity of CO produced in excess
of baseline production was up to 40 times

\[ \text{Injection of MEM cells (5.45\text{g/mHb})} \]

Data from experiments in which erythrocytes damaged with N-ethylmaleimide (NEM) were
injected during pumping of blood in an extracorporeal circuit (one animal) or immediately
after pumping (one animal). Top: Changes in blood radioactivity following injection of the
damaged cells, which were tagged with 51Cr. Bottom: Increases in body CO stores. The
rate of sequestration of the damaged cells by the reticuloendothelial system (signified by a
drop in blood radioactivity) was normal when they were injected after pumping but
slowed when they were injected during pumping. Note the striking increase in the rate of
sequestration of the damaged cells following pumping when the cells were injected during
pumping. Carbon monoxide was presumably produced as a result of catabolism of the
hemoglobin in mechanically damaged red blood cells as well as in the injected, damaged
erythrocytes. Specific activity at the time of injection was taken as 100%, assuming that none
of the radioactivity left the circulation. Broken lines are extrapolations of baseline CO pro-
duction.
Effect of severe hypoxia on the rate of CO production. Inspired gas \( P_{O_2} \) was 150 mm Hg during the first 2 hours of this experiment and then decreased to \(< 50\) mm Hg during the final 2 hours. Note that venous blood \(^{14}C\)CO radioactivity decreased during hypoxia, but the rate of increase of body stores (rate of CO production) did not change. This figure illustrates loss of \(^{14}C\)CO and CO from blood during hypoxia, which has been shown to result from shifting of CO from blood into muscle (6).

It is very unlikely that we have grossly underestimated rates of efflux of hemoglobin. A 2- to 36-fold increase in plasma hemoglobin catabolism would be required to explain the difference between the increase in CO production and the rate of loss of plasma hemoglobin in the present studies. As discussed below, possible error in the calculation of the plasma hemoglobin compartment could explain only a small portion of this difference. In the pump experiments, plasma hemoglobin concentrations as high as 79.3 mg/100 ml were measured (although in most of the animals the plasma hemoglobin concentration did not exceed 50 mg/100 ml), and it is possible that "free" hemoglobin, not bound to haptoglobin, was present. However, previous work (15) has suggested that free hemoglobin is not present in the dog until the plasma hemoglobin level exceeds 80 to 200 mg/100 ml. The basic question relates to the possibility that free hemoglobin may be very rapidly sequestered in the reticuloendothelial system and subsequently catabolized to CO and therefore not accounted for in our calculations based on rates of decrease of plasma hemoglobin or labeled plasma hemoglobin. Very strongly against this possibility are the findings of previous experiments (1, 2) in which solutions containing \(^{14}C\)-hemoglobin or unlabeled hemoglobin were injected intravenously and rates of \(^{14}C\)CO production or increases in CO production were found to be nearly constant and correlated closely with the nearly constant rates of loss of hemoglobin from the plasma space, computed exactly as in the present experiments. Also, if rapid sequestration of free hemoglobin did occur, one would expect the difference between the increase in Vco and the computed rate of loss of hemoglobin from plasma to be larger in experiments with high plasma hemoglobin concentrations and presumably more free hemoglobin, whereas we found (in the pump A experiments) that this difference was independent of the plasma.
MEASUREMENT OF MECHANICALLY INDUCED HEMOLYSIS

357

Furthermore, this difference was noted in experiments in which the peak plasma hemoglobin concentration was less than 10 to 20 mg/100 ml, and it is unlikely that free hemoglobin was present in the plasma.

We have assumed that all of the increase in Vco resulted from catabolism of hemoglobin heme; however, there may have been an increase in catabolism of other hemoproteins that are CO precursors. Evidence is available that hepatic heme is a precursor of CO (16-19), and it is likely that some heme compounds as myoglobin, catalase and cytochrome oxidase are also catabolized to CO. The baseline Vco in anesthetized dogs is considerably greater than can be explained by destruction of erythrocytes (if they have a mean life span of 100 days), suggesting that there are sources of CO other than catabolism of hemoglobin (1). Previous experiments with hemoglobin injection (1) have shown that it is possible to quantify extravascular hemolysis and rates of hemoglobin heme catabolism due to uptake from plasma by measuring changes in Vco even if the baseline Vco is contributed to by heme breakdown from compounds other than hemoglobin. However, pumping of blood in an extracorporeal system may result in alterations that influence extrahemoglobin sources of CO production. Changes in blood flow in peripheral tissues or changes in oxygen uptake may occur (20, 21). Plasma proteins that may be denatured and cleared by the liver (22) may cause alterations in hepatic function (23), and vasopressor agents liberated during pumping may affect function of the liver or other organs (24). However, in the present experiments Vco also increased after infusion of blood that had been pumped in an in-vitro circuit, suggesting that hemodynamic changes caused by veno-arterial perfusion were not responsible for the increase in Vco that occurred in the in-vivo pumping experiments. The fact that Vco did not change in a hypoxic animal seems to exclude tissue hypoxia per se as a significant factor in the increase of Vco in the pump experiments. Infusion of plasma from blood pumped in vitro also had no measurable effect on Vco, indicating that alterations in plasma proteins, changes in vasopressor agents, and other plasma changes did not significantly influence rates of catabolism of body heme. We therefore conclude that the increase in Vco after pumping did not result from catabolism of nonhemoglobin heme and that the CO production not attributable to efflux of hemoglobin from the plasma hemoglobin compartment resulted from extravascular hemolysis.

The quantity of extravascular hemolysis that could explain the high rates of CO production in these experiments was computed as the difference between the total increase in Vco and that explained by loss of hemoglobin from plasma (expressed as μmoles of CO and μmoles of heme) over a 2- to 4-hour period starting immediately after perfusion. We estimated intravascular hemolysis from the total influx of hemoglobin into plasma during pumping, correcting for loss of hemoglobin by assuming that the rate of efflux at this time was the same as that after perfusion. Results of these calculations are presented in Table 3. In the pump A experiments an average of 72.9 ± 8.4% of the total number of erythrocytes destroyed were apparently hemolyzed extravascularly, and in the pump B experiments, an average of 37.2 ± 15.2%.

It is pertinent to discuss possible sources of error in the calculation of extravascular and intravascular hemolysis.

1. Measurement of Vco.—This has been partially discussed under Methods. Increases in Vco could be determined with an error of approximately 10%. Although the experiments with injection of NEM-damaged erythrocytes demonstrated a normal maximal rate of CO production and normal sequestration function of the reticuloendothelial system after pumping, it is still possible that the yield of CO during catabolism of heme (originating from hemoglobin lost from plasma or from damaged erythrocytes destroyed extravascularly) may be reduced due to an alteration of the reticuloendothelial system. This would cause an underestimation of extravascular hemolysis.
2. Calculation of Efflux of Hemoglobin from Plasma.—Error could arise because of loss of hemoglobin into tissues other than the reticuloendothelial system or during determination of the plasma hemoglobin compartment. We consider these errors acceptable, since in the experiments with injection of hemoglobin solution (1, 2) discussed above, CO was produced in the expected ratio to hemoglobin lost from plasma, computed exactly as in the present study. There was no evidence of loss of hemoglobin into urine in the present study, although this does not exclude the possibility of loss into renal or other tissue. The plasma hemoglobin compartment was slightly greater than reported values of plasma volume in anesthetized dogs (25), a fact that may be partially explained by the slight anemia found in some of our experimental animals. Although our determinations were made with dilution of $^{51}$Cr-hemoglobin, elution of the tag should not have had a significant effect in the 15-minute measurement period. The control experiments with hemoglobin injection described in Methods gave similar values for the plasma hemoglobin compartment determined with nonlabeled hemoglobin. If there was error in the calculation of the plasma hemoglobin compartment, it would have only a small effect on the calculation of extravascular hemolysis. Overestimation by 20% would affect extravascular hemolysis less than 3% in the pump A experiments and 10% in the pump B experiments. This small effect is our justification for assuming a value for the plasma hemoglobin compartment in those experiments in which $^{51}$Cr-labeled hemoglobin was not given.

3. Calculation of Intravascular Hemolysis.—The correction made for loss of hemoglobin from plasma during pumping may have caused an error in this calculation; however, it is expected that this would be less than 5%.

4. Comparison of Intravascular and Extravascular Hemolysis.—The comparison made in the present study is valid if all of the erythrocytes damaged during pumping, but not completely destroyed, were sequenced and their hemoglobin catabolized to CO within 3 hours. However, the fact that Vco remained markedly elevated during the entire experiment suggests that there were populations of damaged cells that would subsequently undergo extravascular hemolysis. Therefore, extravascular hemolysis was probably grossly underestimated in the sense that we computed all or nearly all intravascular hemolysis that occurred as a result of pumping but only a portion of the extravascular.

Data previously reported in the literature suggest but do not prove that extravascular hemolysis is an important mechanism of erythrocyte destruction after extracorporeal perfusion. Following cardiac surgery with cardiopulmonary bypass, Kusserow et al. (26), Abbott et al. (27), and Brinsfield et al. (28) noted the appearance of anemia that could not be explained by intravascular hemolysis. Hewitt et al. (29) measured erythrocyte survival using $^{51}$Cr-labeled erythrocytes following heart-lung bypass with an oxygenator and observed a normal half-life for 20 days postoperatively, followed by a decrease in erythrocyte survival. Burke and Gardner (30) observed a significant decrease in erythrocyte life span in a similar study. Indeglia et al. (31) reinjected dogs with blood subjected in vitro to pressure, occlusion, shear and wall interactions and noted an increase in erythrocyte destruction. Decreased osmotic fragility following "mechanical trauma" to the erythrocyte has been observed, as well as abnormalities of sodium and potassium transport and membrane peroxidation (32).

The data obtained in the present study suggest that extravascular hemolysis is quantitatively of great significance with regard to erythrocyte destruction during the first 3 hours after perfusion and may be more significant than intravascular hemolysis. No correlation was found between intravascular and extravascular hemolysis in our small series of experiments, suggesting that they may be influenced by different variables when associated with extracorporeal circulation.

The techniques employed in these studies should be useful in determining variables that influence patterns of extravascular and intravascular...
vascular hemolysis during mechanical perfusion or under other conditions in which these processes occur simultaneously.

**Acknowledgment**

The authors gratefully acknowledge the technical assistance of Helene Brooks, Maria Lewkow, Barbara Bruser, Ann Cusack, Constan Anderson and Margaret Strickler, and the encouragement and assistance of Dr. Robert E. Forster.

**References**


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Mechanically Induced Intravascular and Extravascular Hemolysis in Dogs

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Circ Res. 1970;26:347-360
doi: 10.1161/01.RES.26.3.347

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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