Mechanically Induced Intravascular and Extravascular Hemolysis in Dogs

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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 erythrocyte destruction extracorporeal circulation

■ In previous studies in our laboratory (1-3), when hemoglobin solutions or damaged erythrocytes were injected intravenously, rates of carbon monoxide production exceeded baseline rates in a molal ratio to heme catabolism (1 μmole heme yielding 1 μmole CO). Therefore, we thought that measurements of CO production could be used to quantify extravascular and intravascular hemolysis under conditions in which these processes occur simultaneously. We measured CO production in 11 anesthetized dogs before and after pumping blood in an extracorporeal system at rates sufficient to result in plasma hemoglobin concentrations ranging from 1 to 79.3 mg/100 ml. Two different pumps were used in these experiments. We estimated rates of efflux of hemoglobin from plasma and compared them with increases in rates of CO production. We also evaluated the possibility that physiological changes that may occur as a result of pumping blood in an extracorporeal system may cause an increase in the rate of catabolism of nonhemoglobin heme and thus contribute to the rate of endogenous production of carbon monoxide, influence the yield of CO production resulting from hemoglobin catabolism, or alter the ability of the reticuloendothelial system to sequester and destroy damaged erythrocytes.

Methods

Adult male mongrel dogs weighing between 13.0 and 23.2 kg were anesthetized with pentobarbital, 30 mg/kg iv. A tracheostomy was performed through a midline incision, and a snugly fitting endotracheal tube encircled by two heavy ligatures to ensure an airtight connection was inserted. Intravenous fluids (5 g dextrose/

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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 (¼ inch i.d.) and ¼ 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 (⅜ 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])[1] 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 ^{14}CO radioactivity ([^{14}CO]), [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 ^{51}Cr-labeled hemoglobin was injected intravenously at the beginning of the experiment. After 15 minutes a blood sample was collected and the plasma analyzed for ^{51}Cr radioactivity, which was used in calculating the "plasma hemoglobin compartment," i.e., the hemoglobin dilution space. Serial measurements of plasma ^{51}Cr 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. In two experiments erythrocytes were labeled with ^{51}Cr, damaged by incubation with N-ethylmaleimide (NEM), 32 μmoles/ml (5), and reinjected intravenously so that we could measure the ability of the reticuloendothelial system to sequester damaged cells and produce CO. In one experiment blood was pumped in the extracorporeal system with pump A at a rate of 500 ml/min for 60 minutes and the NEM-damaged, tagged cells were injected immediately after cessation of pumping. In the other experiment (using the same pump rate and pumping time), the damaged cells were injected during veno-arterial perfusion. Erythrocyte ^{51}Cr radioactivity

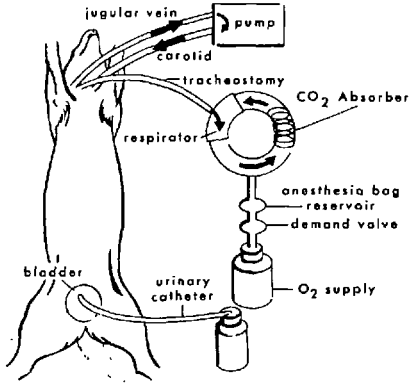

FIGURE 1

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

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

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 20 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 Po_2 of 150 mm Hg, ^{14}CO was administered into the rebreathing system and serial measurements of [COHb] were made over a 2-hour period. The Po_2 of the inspired gas was then decreased to less than 60 mm Hg, and serial measurements of [COHb] and [^{14}CO] were performed during the subsequent 2-hour period. Blood was not pumped in this experiment.

MEASUREMENT OF THE RATE OF CARBON MONOXIDE PRODUCTION

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 ^{14}CO 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. After complete mixing of the administered ^{14}CO in the body stores, blood ^{14}CO radioactivity ([^{14}CO]) also decreased with the pumping procedure. It appeared that these decreases in [COHb] and [^{14}CO] in blood resulted from shifts of CO from blood into extravascular tissue. 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 ^{14}CO to the body stores to compensate for the change in partition of CO between vascular and extravascular CO stores.

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

$$X^*(t) = X^*(0), \quad (1)$$

where $X^*(t)$ and $X^*(0)$ are total body ^{14}CO 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:

$$SA(t) = X^*(t)/X(t), \quad (2)$$

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

Rearrangement of the above equations yields

$$X^*(t) = SA(t) \cdot X(t), \quad (3)$$

and similarly for time zero,

$$X^*(0) = SA(0) \cdot X(0). \quad (4)$$

From equation 1

$$X(t) = \frac{X(0) \cdot SA(0)}{SA(t)}. \quad (5)$$

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 ($\dot{V}co$) by subtracting the baseline $\dot{V}co$ from that determined following extracorporeal circulation. In anesthetized dogs, the baseline $\dot{V}co$ 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 tissue did not change before pumping, we determined the baseline $\dot{V}co$ from the increase in [COHb] alone (4). The error in determining baseline $\dot{V}co$ was ± 0.03 ml/hour; after pumping, the error was slightly greater due to the additional errors of the ^{14}CO measurement. The assumption that the total body ^{14}CO 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 ^{14}CO to $^{14}CO_2$ following pumping in one experiment was only 0.16%/hour of the total body ^{14}CO radioactivity. In addition, the previously mentioned studies

obtained during hypoxemia and shock (8) have shown that the shift of CO into muscle is reversible. The assumption that specific activity is equal in all of the body CO stores is tantamount to saying that mixing of 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 CO has shifted into muscle, mixing of CO is sufficiently rapid (8) that significant error in calculating $\dot{V}co$ 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 ^{51}Cr-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 ^{51}Cr-labeled hemoglobin was prepared by washing dog erythrocytes twice in cold saline and incubating them with $Na_2{}^{51}CrO_4$ 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 ^{51}Cr-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 and 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 [COHb] was measured by an infrared method (11). Blood [^{14}CO] was determined in an ionization chamber after extraction from 2 ml of blood (8). ^{51}Cr 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 CO production did not differ significantly from those obtained in a previous

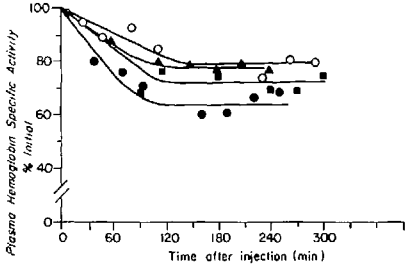

FIGURE 2

Specific activity of plasma hemoglobin following simultaneous intravenous injections of hemoglobin labeled with ^{51}Cr and unlabeled hemoglobin 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.

TABLE 1

Baseline Data

Dog no.	Wt (kg)	Plasma Hb conen (g/100 ml)	Baseline $\dot{V}_{CO}$ (ml/hr)	Pump rate (ml/min)	Pump time (min)	Plasma Hb compartment (ml/kg)
			Pump A Experiments			
1	15.4	11.98	0.18	77	135	76
2	14.5	13.87		485	85	
3	15.4	13.2		300	60	77
4	23.1	14.75		450	65	
5	17.7	13.73	0.11	540	75	77
6	13.0	14.33	0.10	520	90	
7	18.6	11.78	0.12	340	120	75
8	17.2	11.28	0.35	150	60	
MEAN	16.9	13.12	0.17			
SE		0.45	0.04			
			Pump B Experiments			
9	18.2	12.56		440	45	64
10	23.2	16.31		440	15	
11	18.1	10.29	0.20	400	30	
MEAN	19.8	13.05				
SE		1.43				
			Continuous Pump A Experiments			
12	14.5	9.89		725	180	74.6
13	14.5	12.85	0.09	250	165	77.5
14	17.0	14.31	0.12	120	165	60.0
MEAN	15.7	12.35	0.11			
SE		1.32	0.01			

study of anesthetized dogs (1). In every experiment the rate of CO production was greater after pumping than the baseline rate. In most of the experiments it increased significantly during the first hour after pumping, reached a maximum during the second hour, and decreased somewhat in subsequent measurement periods. Maximal rates ranged from 0.25 to 2.0 ml/hour, averaging 0.97 ± 0.22 ml/hour[2] in the pump A experiments and 0.97 ± 0.34 ml/hour in the pump B experiments (Table 2). In ten of the animals CO was still being produced at an increased rate during the final hour of measurement, which in one experiment was 6 hours after cessation of pumping. Data from one of the pump experiments are illustrated in Figure 3.

A decrease in blood [^{14}CO] occurred in nine 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 μmoles heme with pump A and from 42.7 to 64.1 μmoles heme

[2]Throughout this paper the ± values are standard errors of the means.

TABLE 2

Effect of Pumping on Plasma Hemoglobin Catabolism and CO Production

Dog no.	Plasma Hb concentration		Highest $\dot{V}_{CO}$* (ml/hr)	Measurement period			Contribution of plasma Hb catabolism to excess CO (%)
	Highest (mg/100 ml)	Rate of decrease (mg/100 ml/hr)		Time (min)	Plasma Hb loss (μmoles heme)	Excess CO produced (μmoles)	
			Pump A Experiments				
1	8.3	1.0	1.70	180	1.9	73.6	2.7
2	21.0	1.5	2.00	120	18.8†	178.7	10.5
3	26.0	1.0	0.25	180	1.9	10.7	18.2
4	46.0	0.0	0.35	240	0	38.9	0
5	55.0	5.0	1.15	180	14.1	104.9	13.4
6	46.5	4.0	0.75	180	9.4†	87.1	10.7
7	17.5	2.3	0.53	180	6.2	66.9	9.3
8	1.0	0.0	1.00	180	0	35.7	0
MEAN	27.7	1.9	0.97		6.5	74.6	8.1
SE	6.9	0.6	0.22		2.5	18.3	2.3
			Pump B Experiments				
9	79.3	7.1	0.70	180	28.5	55.8	51.0
10	61.0	4.5	0.42	180	15.8†	20.5	77.0
11	55	4.1	1.80	180	49.4†	157.2	31.4
MEAN	65.1	5.2	0.97		31.2	77.8	53.1
SE	6.0	0.8	0.34		7.7	33.4	10.7
			Continuous Pump A Experiments				
12	38.4		0.34				
13	17.6		0.76				
14	7.8		0.40				
MEAN	21.3		0.50				
SE	9.0		0.13				

*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 ^{51}Cr-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 2- to 4-hour postperfusion period. In each of the pump experiments the quantity of CO

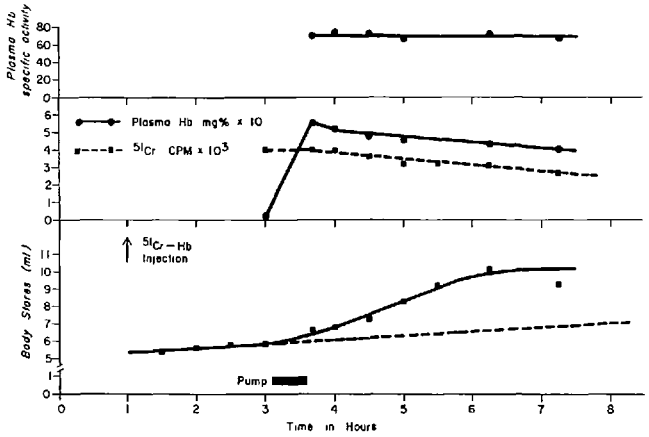

FIGURE 3

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 ^{51}Cr-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 3

Intravascular and Extravascular Hemolysis

Dog no.	IH (μmoles heme)	EH (μmoles heme)	Time of EH (min)	EH/total hemolysis (%)	IH/EH
		Pump A Experiments			
1	5.8	71.7	180	92.5	0.08
2	13.4	159.9	120	92.3	0.08
3	18.1	8.8	180	32.7	2.06
4	48.2	38.9	240	44.7	1.24
5	44.1	90.8	180	67.3	0.49
6	27.4	77.7	180	73.9	0.35
7	14.4	60.7	180	80.8	0.24
8	0.0	35.7	180	100.0	0.00
MEAN	21.4	68.0		73.0	0.57
SE	6.1	16.1		8.5	0.25
		Pump B Experiments			
9	54.0	27.3	180	33.6	1.98
10	64.1	4.7	180	6.8	13.64
11	42.7	107.8	180	71.6	0.40
MEAN	53.6	46.6		37.3	5.34
SE	6.2	31.3		18.8	2.56

IH = intravascular hemolysis; EH = extravascular hemolysis.

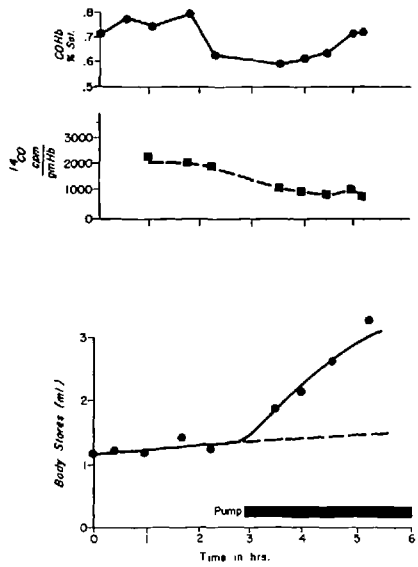

FIGURE 4

Data obtained in a constant-pumping experiment. This plot illustrates measurements of blood ^{14}CO radioactivity ([^{14}CO]) 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.

produced in excess of baseline production was much larger than could be accounted for by the quantity of heme catabolized as a result of loss of hemoglobin from plasma. In the pump A experiments an average of only 8.1 ± 2.3% of the excess CO could be explained by catabolism of plasma hemoglobin, and in the pump B experiments, an average of 53.1 ± 10.7%.

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 18 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 ($t^{\frac{1}{2}}$ 39 minutes), but after cessation of pumping the rate of disappearance from circulating blood increased markedly ($t^{\frac{1}{2}}$ 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 $\dot{V}_{CO}$ 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 $\dot{V}_{CO}$ was 0.42 ml/hour, and $\dot{V}_{CO}$ 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 $\dot{V}_{CO}$ of only 0.04 ml/hour, which is not detectable. These findings suggest that the increase in $\dot{V}_{CO}$ 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, $\dot{V}_{CO}$ did not change significantly during the 2-hour period when arterial P_{O_2} was maintained between 35 and 50 mm Hg, compared to the baseline $\dot{V}_{CO}$ (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

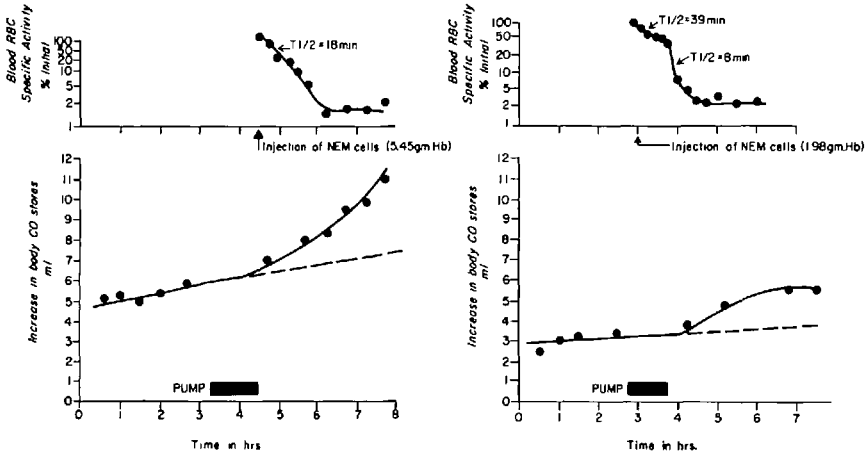

FIGURE 5

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* ^{51}Cr. **Bottom:** *Increases in body CO stores. The rate of sequestration of the damaged cells by the reticuloendothelial system (signified by a decrease 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 production.*

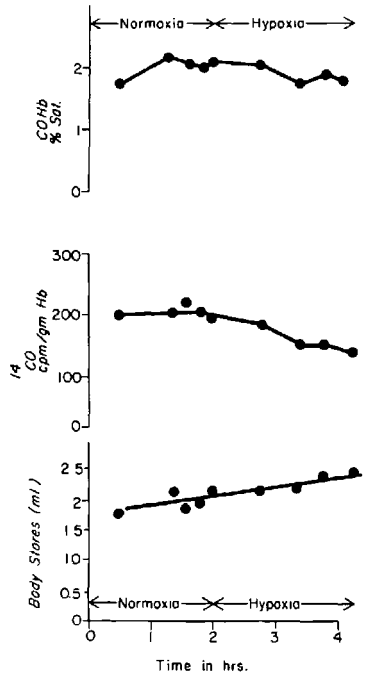

FIGURE 6

Effect of severe hypoxia on the rate of CO production. Inspired gas Po_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}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}CO and CO from blood during hypoxia, which has been shown to result from shifting of CO from blood into muscle (6).

greater than could be explained by catabolism of hemoglobin leaving the plasma. Since there is no evidence that CO production occurs at a significant rate in the dog or in man from processes other than catabolism of heme (14), this finding suggests that rates of heme catabolism were much greater than those calculated from rates of efflux of hemoglobin from plasma. It seems very likely that this increased heme catabolism resulted from catabolism of hemoglobin in erythrocytes that were traumatized and subsequently removed from the circulation. However, other possible explanations must be considered.

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}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 $\dot{V}co$ 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

hemoglobin concentrations. 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 $\dot{V}_{CO}$ 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 such heme compounds as myoglobin, catalase and cytochrome oxidase are also catabolized to CO. The baseline $\dot{V}_{CO}$ 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 $\dot{V}_{CO}$ even if the baseline $\dot{V}_{CO}$ 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 $\dot{V}_{CO}$ 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 $\dot{V}_{CO}$ that occurred in the in-vivo pumping experiments. The fact that $\dot{V}_{CO}$ did not change in a hypoxic animal seems to exclude tissue hypoxia per se as a significant factor in the increase of $\dot{V}_{CO}$ in the pump experiments. Infusion of plasma from blood pumped in vitro also had no measurable effect on $\dot{V}_{CO}$, 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 $\dot{V}_{CO}$ 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 $\dot{V}_{CO}$ 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 $72.9 \pm 8.4\%$ of the total number of erythrocytes destroyed were apparently hemolyzed extravascularly, and in the pump B experiments, an average of $37.2 \pm 15.2\%$.

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

1. *Measurement of* $\dot{V}_{CO}$.—This has been partially discussed under Methods. Increases in $\dot{V}_{CO}$ 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 2% 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 sequestered and their hemoglobin catabolized to CO within 3 hours. However, the fact that $\dot{V}co$ 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 intra-

vascular hemolysis during mechanical perfusion or under other conditions in which these processes occur simultaneously.

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