

## THE FORMATION OF CARBON MONOXIDE DURING PEROXIDATION OF MICROSOMAL LIPIDS

David G. Wolff and Wayne R. Bidlack  
Department of Pharmacology  
University of Southern California  
School of Medicine  
Los Angeles, California 90033

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**SUMMARY:** The evolution of carbon monoxide during lipid peroxidation has been demonstrated in microsomal membranes. The formation of carbon monoxide was dependent on the peroxidation process, but independent of the initiators (NADPH-ADP·Fe<sup>+3</sup> or Ascorbate·Fe<sup>+3</sup>) used. Contrary to published results, the carbon monoxide does not result from heme catabolism. Carbon monoxide was generated during the peroxidation of isolated phospholipids, indicating that carbon monoxide may form directly during the peroxidative degradation of unsaturated fatty acids.

Hepatic microsomes undergo enzymatic peroxidation of membrane lipids in the presence of NADPH, oxygen, iron in either the ferric or ferrous form, and certain chelators, i.e., ADP (1,2). Nishibayashi, *et al.* (3) have noted the generation of carbon monoxide (CO) during this peroxidative process. Repeating spectral scans of the NADPH-iron peroxidizing microsomal membranes revealed the formation of an absorbing complex at 450 nm (P<sub>450</sub>-CO). Schacter, *et al.* (4,5) suggested that the CO generated could result from catabolism of heme, as in the case of heme oxygenase (6). However, they concluded the CO generated was independent of the heme oxygenase activity, but was related to destruction of heme during the peroxidation process. Levin, *et al.* (7) used only NADPH, without added iron, and followed peroxidation over a longer period of time, 30 minutes. Both laboratories concluded that the amount of CO formed corresponded directly to the amount of heme destroyed during the peroxidation process.

In this paper, we provide evidence that CO is generated from peroxidizing lipids independent of heme destruction. It seems likely that heme loss occurs when cytochrome P<sub>450</sub> participates as a peroxidase in the metabolism of the lipid hydroperoxides.

**METHODS:** Rat liver microsomes were prepared daily by minor modifications of the procedure of Ernster, *et al.* (8). The microsomal pellet was collected from a 28,000 g supernatant fraction by centrifugation at 105,000 g

for 60 minutes. To remove residual hemoglobin contaminate, the microsomes were resuspended in Tris (25 mM) - KCl (150 mM) buffer, pH 7.4 and recentrifuged at 105,000 g for 60 minutes. The surface of the pellet was thoroughly rinsed and the microsomes were resuspended in Tris (25 mM) - KCl (150 mM) buffer, pH 7.4. Protein was determined by the method of Lowry, *et al.* (9). Incubations were carried out at 22-25°C unless otherwise stated. Oxygen was measured polarographically with a Clark-type electrode.

Lipid peroxidation products were determined by the 2-thiobarbituric acid assay described previously (10). The optical densities were recorded at 532 nm and the concentration of malonaldehyde was determined using a millimolar extinction coefficient of 156.

All spectra were recorded using an Aminco DW-2 spectrophotometer in the split beam mode. Cytochrome  $b_5$  was assayed spectrophotometrically by measuring the dithionite-reduced minus oxidized difference spectrum, using the extinction between 424 and 409 nm as  $185 \text{ mM}^{-1}\text{cm}^{-1}$  (11). Cytochrome  $P_{450}$  was measured by difference spectrophotometry by the method of Omura and Sato (11) using an extinction coefficient between 450 and 490 nm of  $91 \text{ mM}^{-1}\text{cm}^{-1}$ .

Total microsomal heme was determined by the method of Falk, *et al.* (12) which measures the pyridine hemochromogen. The difference spectra between 557 and 575 nm was taken and an extinction of  $32.4 \text{ mM}^{-1}\text{cm}^{-1}$  was used (13).

Carbon monoxide was measured during enzymatic peroxidation by repeated spectral scans of the NADPH-ADP- $\text{Fe}^{+3}$  microsomal suspension recorded against an NADPH-reduced microsomal reference. The reduced nucleotide maintained the sample cytochrome  $P_{450}$  in a reduced state and as CO was generated a  $P_{450}$ -CO complex was formed.

To quantitate the amount of CO formed during the reaction, hemoglobin was added to bind the CO as carboxyhemoglobin. EDTA (200  $\mu\text{M}$ ) was added to both the sample and the reference to stop the peroxidation reaction, and to assure a distinct absorbance maximum for the hemoglobin-CO measurement. Hemoglobin (4  $\mu\text{M}$ ) was gently mixed into the sample and reference. One minute was allowed to assure maximum CO binding and the difference spectra was recorded. Using the optical density at 421 nm minus the isobestic point at 480 nm, an extinction coefficient of  $58.7 \text{ mM}^{-1}\text{cm}^{-1}$  was determined and used to quantitate the amount of carboxyhemoglobin formed and hence the amount of CO generated.

Hepatic phospholipids were isolated by the method of Folch, *et al.* (14) using chloroform-methanol (2:1, v:v). The chloroform layer was collected and taken to dryness under nitrogen. The phospholipids were then sonicated into a micellar suspension (0.5 mg/ml) in Tris (25 mM) - KCl (150 mM) buffer, pH 7.4, containing ascorbic acid (0.1 mM). Lipid peroxidation was initiated by the addition of  $\text{FeCl}_3$  (6  $\mu\text{M}$ ) and the samples were incubated at 37°C. Non-enzymatic peroxidation of the microsomal membrane was initiated by the same concentrations of ascorbate and iron.

**RESULTS:** Animal variance accounts for large standard deviations in reporting the mean values of various cytochrome and heme assays. However, the data presented here are based on triplicate assays for each data point.

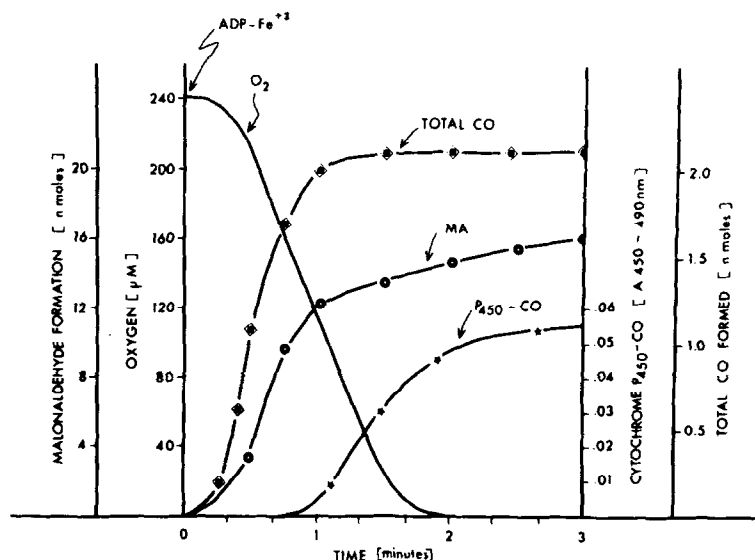


Figure 1. The generation of CO during microsomal enzymatic peroxidation. A final volume of 1.0 ml Tris (25 mM) - KCl (150 mM), pH 7.4, buffer contained: microsomes, 1.0 mg; NADPH, 250  $\mu$ M; ADP, 0.5 mM; and  $\text{FeCl}_3$ , 20  $\mu$ M. The reaction was initiated by the addition of the  $\text{ADP} \cdot \text{Fe}^{+3}$  complex.

The same microsomal preparation, made from two or more rats, was used to compile each Table or Figure presented.

In Figure 1, the generation of CO is indicated in relation to other parameters of the peroxidation process, including oxygen consumption and malonaldehyde (MA) formation. The microsomal suspension was reduced with NADPH and the peroxidation reaction was initiated by the addition of  $\text{ADP} \cdot \text{Fe}^{+3}$ . Repeating spectral scans revealed the formation of an absorbing complex at 450 nm (Figure 1,  $\text{P}_{450}\text{-CO}$  curve). As described by others (3,4,5,7), this complex is the result of CO being generated and binding to the NADPH reduced cytochrome  $\text{P}_{450}$ . These earlier reports made calculations of the total concentration of CO present from the CO binding constant known for cytochrome  $\text{P}_{450}$ .

The time lag in the formation of the  $\text{P}_{450}\text{-CO}$  spectra (Figure 1) may result from oxygen competition, which allows the spectra to develop only as the system becomes anaerobic. At three minutes, the  $\text{P}_{450}\text{-CO}$  absorbance

TABLE I. Heme Loss and CO Formation During Lipid Peroxidation of the Microsomal Membrane<sup>a</sup>

Experiment Number	Reaction Time (min.)	Cytochrome b <sub>5</sub> (nmoles/mg)	Cytochrome P <sub>450</sub> (nmoles/mg)	Total Heme <sup>b</sup> (nmoles/mg)	Heme Loss (nmoles)	Total CO Formed (nmoles)
1.	0	0.61	0.79	1.61	----	----
	3	0.61	0.79	1.42	0.19	2.15
2.	0	0.54	0.76	1.80	----	----
	3	0.54	0.76	1.30	0.50	3.29
3.	0	0.77	1.19	2.22	----	----
	3	0.77	1.01	2.09	0.13	2.07
4.	0	0.56	0.61	1.53	----	----
	3	0.56	0.61	1.19	0.34	1.87

<sup>a</sup>Reaction conditions are the same as described in Figure 1.

<sup>b</sup>Total heme exceeded the predicted sum of cytochrome b<sub>5</sub> and cytochrome P<sub>450</sub> in every case. Hemoglobin contamination may influence the total (0 to 0.06 nmoles hemoglobin/mg was detected in various microsomal preparations).

indicates approximately 0.5 nmoles of CO bound to cytochrome P<sub>450</sub>. However, when we carefully reduced the system further by addition of dithionite, all of the P<sub>450</sub> (ca. 1 nmole) present had CO bound to it. The limit then in measuring the total CO present was the total amount of P<sub>450</sub> available! By utilizing hemoglobin to detect the presence of CO, we have been able to quantitate the total CO present in our reaction system at any time interval (see Methods). To our surprise, instead of finding 0.5 to 1.0 nmoles of total CO from the P<sub>450</sub> spectra, we have found a total of 2.1 nmoles in this system (Figure 1) and as much as 3.0 nmoles in other microsomal preparations. In all experiments, the amount of CO exceeded the total amount of heme present and made the source of the CO open to question.

Interestingly, under the same reaction conditions, only a portion of the total heme was lost. Thus, we did not obtain a molar ratio of one to one for heme loss and CO formation (Table I) as previously reported (4,5). Cytochrome b<sub>5</sub> remains unaffected by the peroxidation process and cytochrome P<sub>450</sub> loss was variable, but never in excess of 30%. This imbalance of heme loss and CO formation suggested an alternative source for CO generation

TABLE II: Dependence of CO Generation on Lipid Peroxidation<sup>a</sup>

Experiment Number	Reaction System	Inhibitor <sup>b</sup>	Total O <sub>2</sub> Consumed in 3 minutes (nmoles)	Total CO Formed in 3 minutes (nmoles/mg)	Total Heme Loss in 3 minutes (nmoles/mg)
1.	NADPH + ADP·Fe <sup>3+</sup>	--	240	2.15	0.22
2.	NADPH	--	12	0.00	0.00
3.	NADPH + ADP·Fe <sup>3+</sup>	EDTA@15 sec	30	0.18	0.06
4.	NADPH + ADP·Fe <sup>3+</sup>	EDTA@30 sec	125	1.09	0.21

<sup>a</sup>Reaction conditions are the same as described in Figure 1.

<sup>b</sup>A final concentration of EDTA (200 μM) was added at the designated time following initiation of peroxidation.

TABLE III: Generation of CO Independent of the Agent Used to Initiate Peroxidation

Peroxidizable Substrate	Experiment Number	Initiator	Reaction		Peroxidation (nmoles MA/cc)	CO Formation <sup>c</sup> (nmoles/cc)
			Time (min)	Temperature (°C)		
<u>Microsomes</u> <sup>a</sup>	1.	NADPH	3	37	0.72	0.00
			30	37	5.11	0.29
	2.	NADPH+ADP·Fe <sup>3+</sup>	1	25	10.40	1.65
			3	25	21.20	2.30
	3.	Ascorbate·Fe <sup>3+</sup>	1	37	1.60	0.00
			3	37	6.90	1.00
<u>Phospholipids</u> <sup>b</sup>	1.	Ascorbate·Fe <sup>3+</sup>	60	37	4.60	0.59
	2.	Ascorbate·Fe <sup>3+</sup>	60	37	3.40	0.48

<sup>a</sup>Microsomes (1.0 mg/cc) were incubated for the indicated time in Tris (25 mM) - KCl (150 mM), pH 7.4 buffer containing the appropriate initiators: NADPH (250 μM), ADP (0.5 mM) - FeCl<sub>3</sub> (20 μM), Ascorbate (0.1 mM) - FeCl<sub>3</sub> (6 μM).

<sup>b</sup>Phospholipids (0.5 mg/cc) were incubated as a micellar suspension under similar conditions.

<sup>c</sup>At zero time, no CO was measurable.

must be present. Since, even if we assumed all four methene bridges were oxidized to CO, the total does not equal the amount of CO formed.

As indicated in Table II, formation of CO was directly dependent upon the extent of lipid peroxidation. When EDTA was added to stop enzymatic

peroxidation at various time intervals, total oxygen consumption is decreased and the formation of CO was restricted proportionately. In addition, CO was formed even when peroxidation was initiated non-enzymatically by ascorbate and iron (Table III). In the absence of NADPH, the ascorbate system indirectly confirmed the non-participation of microsomal heme oxygenase.

Most important, however, was the demonstration that CO was generated during peroxidation of isolated hepatic phospholipids (Table III). Even though the CO formation was slower, the generation of CO independent of heme indicates CO can be formed during the peroxidative degradation of membrane lipids. The difference in rate may only reflect differences in the lipid structure in the micelle compared to their structure in the membrane.

DISCUSSION: The formation of carbon monoxide during enzymatic peroxidation of the microsomal membrane has been credited to the catabolism of heme proteins, specifically cytochrome P<sub>450</sub> (4,5,7). The proposed mechanism being analogous to that described for heme oxygenase (6). The oxidation of the  $\alpha$ -methene bridge causes the opening of the ring structure and release of CO. However, even though this enzyme is present in the microsomal membrane, it does not appear to be an active participant in generating CO during membrane peroxidation.

Formation of CO is directly dependent on the extent of membrane peroxidation. When peroxidation is inhibited, CO accumulation decreases proportionately. In addition, independent of the initiator used, NADPH-ADP·Fe<sup>+3</sup> or Ascorbate·Fe<sup>+3</sup>, CO is formed during peroxidation.

Other laboratories (4,5,7) have reported a one to one molar ratio for the total CO formed compared to the amount of heme destroyed. We have always found more CO formed than heme destroyed. This variance suggested that an alternative source of CO must be present, such as the peroxidizing lipids. Indeed, when isolated phospholipids were peroxidized, CO was produced.

The loss of total heme was generally reflected as a specific decrease in cytochrome  $P_{450}$ . The variability in the decrease of heme reported by us and by others (4,5,7) may result from differences in sampling times. During peroxidation, lipid hydroperoxides accumulate in the reaction system and may cause: a) a decreased binding capacity of  $P_{450}$  for CO as noted by Hrycay and O'Brien (15) or b) actual destruction of the heme during metabolism of the hydroperoxides (17,18) by cytochrome  $P_{450}$ . The peroxidase activity of cytochrome  $P_{450}$  has been discussed previously (15,16).

Thus, we suggest that CO formation is independent of heme loss, and that the membrane lipids undergoing peroxidative degradation act as a source of CO. The specific phospholipids and fatty acids involved in CO production are currently being evaluated.

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