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Chemical Evidence for Production of Hydroxyl Radicals during Microsomal Electron Transfer

Abstract. *Rat liver microsomes generate methane from dimethyl sulfoxide and ethylene from either methional or 2-keto-4-thiomethylbutyric acid during electron transfer initiated by reduced nicotinamide-adenine dinucleotide phosphate (NADPH). Hydrocarbon gas production is suppressed by hydroxyl radical scavenging agents. Azide, an inhibitor of catalase, augments the production of hydrocarbon gases. These observations constitute chemical evidence for the generation of hydroxyl radicals by microsomes.*

Reduction of oxygen during microsomal electron transfer reactions has been the subject of many investigations. Hydrogen peroxide has been identified as an end product (*1*) and the superoxide radical as an intermediate (*2*). Iron-peroxy complexes have also been implicated (*3*). Further knowledge of products or intermediates of oxygen metabolism could help to elucidate aspects of microsomal function and of cytochrome P-450 mediated reactions.

We now report chemical evidence for the formation of hydroxyl radicals (·OH) during microsomal electron transfer. Generation of hydroxyl radicals is not unexpected in systems that generate H_2O_2 (Eq. 1)

$$H_2O_2 + \text{iron catalyst} \rightarrow \cdot OH \quad (1)$$

particularly when catalysts such as iron or iron chelates are present (*4*). Cytochrome P-450, an endogenous heme protein in microsomes, may play an important role and deserves further investigation. In our experiments, no external iron was added.

Three agents were used to detect hydroxyl radicals: methional (3-thiomethylpropanal), 2-keto-4-thiomethylbutyric acid (KTBA, the keto-acid analog of methional), and dimethyl sulfoxide (DMSO). These agents scavenge hydroxyl radicals and form ethylene gas from methional or KTBA and methane gas from DMSO. Generation of ethylene has been used to detect hydroxyl radicals, for example, during the xanthine-xanthine oxidase reaction (*5*), the autoxidation of certain organ-specific cell toxins (*6*), and the phagocytic process (*7*). Dimethyl sulfoxide reacts with hydroxyl radicals to generate methyl radicals ($\cdot CH_3$) (*8*), which give rise to methane by hydrogen abstraction. Methane generation from DMSO during oxidant hemolysis of vitamin E-deficient rat erythrocytes has been reported (*9*). The reactions involved in the formation of hydrocarbon gases from methional, KTBA, and DMSO can be summarized by Eqs. 2 to 4, respectively (*5*, *8*, *10*):

$$CH_3SCH_2CH_2CHO + \cdot OH \rightarrow CH_2{=}CH_2 + HCOOH + {}^1/_2(SCH_3)_2 \quad (2)$$

$$CH_3SCH_2CH_2COCOOH + \cdot OH \rightarrow CH_2{=}CH_2 + 2CO_2 + {}^1/_2(SCH_3)_2 \quad (3)$$

$$CH_3SOCH_3 + \cdot OH \rightarrow \cdot CH_3 + CH_3SOOH \quad (4)$$

Liver microsomes were prepared from male Sprague-Dawley rats (*11*), washed once, and suspended in 125 m*M* KCl. All solutions were prepared with Millipore water subsequently distilled in an all-glass apparatus. Microsomes were incubated at 37°C in a medium consisting of 83 m*M* potassium phosphate buffer (*p*H 7.4), 10 m*M* potassium pyrophosphate, 10 m*M* $MgCl_2$, 0.3 m*M* $NADP^+$, 0.5 m*M* azide, 10 m*M* glucose-6-phosphate (G-6-P), and 7 units of glucose-6-phosphate dehydrogenase (G-6-PD) in a total volume of 3 ml. The $NADP^+$, G-6-P, and G-6-PD functioned as an NADPH-generating system. Drug metabolism as well as ethanol oxidation by microsomes requires NADPH (*12*), whose role appears to be reduction of cytochrome P-450 from the ferric to the ferrous state. The role of azide was to inhibit microsomal catalase. Samples were prepared in the cold and microsomes (approximately 7.5 mg of protein) were added. The $NADP^+$ was added last to initiate the reaction, and the samples were then placed into a water bath at 37°C. Samples were contained in sealed 18-ml screw cap tubes. The caps were fitted with silicone septa, which were punctured for removal of a sample of gas phase. A gas-tight, plastic and rubber, disposable 1 ml syringe (Becton-Dickinson) was used. Before removing a sample of gas, the plunger was drawn back and forth ten times to ensure that the gas content of the tube was adequately mixed. The gas sample (0.5 ml) was then injected directly into a Hewlett-Packard model 5750 gas chromatograph for measurement of the hydrocarbon gases (*6*, *13*).

Table 1 shows that time-dependent production of ethylene was observed with either methional or KTBA as substrate. Methane production was observed with DMSO. The yield of methane was less than that of ethylene. In control studies, no hydrocarbon gases were generated when G-6-P, G-6-PD, or $NADP^+$ was omitted. Therefore, the generation of NADPH was an absolute requirement. None of the latter constituents, either alone or together, generated hydrocarbon gases in the absence of microsomes. Therefore, an absolute requirement for microsomes was demonstrated, and hydrocarbon gas production could not be attributed directly to the NADPH-generating system or to an interaction between this system and the buffer. Lastly, no gas was formed when methional, KTBA, or DMSO was omitted. Thus, the appearance of ethylene or methane required the presence of substrate and the simultaneous initiation of microsomal electron transfer reactions.

Ethanol and *n*-butanol are good scavengers for hydroxyl radicals, whereas urea is not (*13*, *14*). When either ethanol or *n*-butanol was added, the formation of hydrocarbon gas was suppressed. For example, 50 m*M* ethanol suppressed eth-

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ylene production from KTBA (10 m*M*) by 46 percent ($N = 8$, $P < .001$). Urea failed to suppress ethylene production. These observations are consistent with the scavenging properties of the added substances.

In the absence of azide, H_2O_2 cannot accumulate because isolated microsomes are often contaminated with catalase, which degrades H_2O_2. In the presence of azide, which inhibits catalase, H_2O_2 accumulates (*15*). When azide was omitted from the reaction medium, the yield of hydrocarbon gas was significantly diminished but not eliminated. For example, in the absence of azide the yield of ethylene from 10 m*M* methional was 44 percent of that in the presence of azide ($N = 6$, $P < .001$, Student's *t*-test). These results are consistent with a need for H_2O_2 as the precursor of hydroxyl radicals.

Because methional, KTBA, and DMSO are good hydroxyl radical scavengers, they would be expected to suppress microsomal reactions that derive from hydroxyl radicals. It has been suggested (*16*) that microsomal oxidation of primary aliphatic alcohols to their corresponding aldehydes is dependent on the generation of hydroxyl radicals. DMSO and other scavengers (mannitol, benzoate, thiourea) have been observed to competitively inhibit the oxidation of ethanol or *n*-butanol in the presence of azide (*16*). Under the conditions of our study, 10 m*M* methional and 10 m*M* KTBA each suppressed the formation of acetaldehyde (*17*) from 33 m*M* ethanol by 48 percent ($N = 6$ to 10; $P < .001$).

The results reported here provide chemical evidence for the generation of hydroxyl radicals by microsomes. Three hydroxyl radical scavengers were metabolized to appropriate products by microsomes; product formation was enhanced when the decomposition of H_2O_2 by catalase was blocked; and competitive scavengers (ethanol, *n*-butanol) suppressed product formation, whereas a nonscavenger (urea) did not. Previously, evidence for hydroxyl radical production by microsomes during electron transfer reactions was adduced when iron (12 to 22 μM) in conjunction with chelators such as adenosine diphosphate (ADP) (*18*) or EDTA (*19*) was added. Fong *et al.* (*18*), used a biologic detection system consisting of isolated lysozomes and studied lipid peroxidation and lysis of lysozomes in the presence of iron-ADP. Lai and Piette (*19*) used a physical method, electron spin resonance in conjunction with spin-trapping agents, to detect ·OH production by microsomes in the presence of iron-EDTA; the hyperfine splitting constants of the spectra permitted ·OH to be distinguished from other possible radicals. Our results with chemical detection systems in the absence of added iron chelates (*20*) appear to verify that hydroxyl radicals are generated during microsomal electron transfer reactions initiated by NADPH.

Table 1. Production of hydrocarbon gases by rat liver microsomes. Values are means and standard deviations for three or four separate microsomal preparations for each substrate.

Substrate	Product	Amount of product (nmole) in	
		12 min	24 min
Methional (10 m*M*)	Ethylene	22.3 ± 5.8	51.1 ± 6.2
KTBA (10 m*M*)	Ethylene	21.0 ± 6.4	45.3 ± 9.6
DMSO (33 m*M*)	Methane	1.0 ± 0.5	3.5 ± 0.7

In addition to ·OH, certain other radicals (alkoxy and peroxy radicals) may produce ethylene from methional (*21*). In view of the complex nature of microsomes, a role for such radicals cannot be excluded. However, in view of the dependence on H_2O_2 and the observations of Lai and Piette (*19*), it is probable that what we observed indirectly by hydrocarbon gas formation is the hydroxyl radical. It has been suggested that singlet oxygen (*22*), an electronically activated state of oxygen, can react with methional to produce ethylene. However, ethylene production was inhibited by ethanol and *n*-butanol, which are essentially unreactive with singlet oxygen (*23*). Nonetheless, a contributory role for singlet oxygen cannot be excluded at this time.

The main importance of our observations is that they identify hydroxyl radicals and provide a means for their future study in microsomal reactions. Hydroxyl radicals formed in intact liver cells could have either damaging or beneficial actions. Because they are cytotoxic, it appears reasonable to hypothesize that they may mediate or promote the actions of some hepatotoxins. On the other hand, their role in the catabolism of some xenobiotic agents requires evaluation (*24*). The study reported here supports a role (*16*) for hydroxyl radicals in the microsomal oxidation of alcohols.

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24. Classical drug metabolic pathways, such as aniline hydroxylation and aminopyrine demethylation, are not blocked by mannitol or benzoate (*16*), two powerful ·OH scavengers. Our study shows that at least one new drug, DMSO, in addition to primary aliphatic alcohols, can be metabolized by microsomes. Methional and KTBA are also metabolized by microsomes. Therefore, further study of the role of ·OH in the metabolism of other xenobiotic agents (such as other sulfoxides) is warranted.
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Microbial Autotrophy: A Primary Source of Organic Carbon in Marine Sediments

Abstract. *The chemoautotrophic fixation of carbon dioxide by bacteria is responsible for an appreciable component of the organic carbon in a sulfide-rich marine mud. A peak of carbon dioxide fixation (at 40 centimeters subbottom) coincides with peaks in the organic carbon content, the ratio of carbon to nitrogen, and bacterial cell counts. Stimulation of fixation by thiosulfate and inhibition by anaerobic conditions implicate the chemoautotrophic sulfur bacteria as primary producers in this environment.*

The existence of autotrophic bacteria capable of synthesizing organic carbon by the reduction of CO_2 is well documented (*1*). However, their effect on the marine ecosystem is poorly understood despite their importance as the only primary producers of organic carbon other than the algae. We report here the first evidence indicating that microbial chemoautotrophs are generating appreciable quantities of organic carbon in marine sediments.

We analyzed acidified and dried subsamples of a diver-collected core from the sulfide-rich muds of Halifax Harbor, Nova Scotia, for their organic carbon content and their ratio of carbon to nitrogen, using a carbon-hydrogen-nitrogen analyzer (Hewlett-Packard 185) (precision of the carbon determination, ± 0.005 percent of the dry sediment weight). A peak of organic carbon is evident at 40 cm subbottom (Fig. 1), and a corresponding peak in the carbon/nitrogen ratio indicates that the organic material at this depth is made up of carbon-rich compounds. In addition, direct counts of bacteria made with an epifluorescent staining method (*2*) show a peak of total (living and dead) cell numbers at the same depth (Fig. 1).

A separate set of samples from the same depth intervals in the core were incubated aerobically, in darkness and at in situ temperature (2°C) for 15 days with 0.11 μM $NaH^{14}CO_3$ (New England Nuclear). The macromolecular products of the incubation were precipitated with trichloroacetic acid, and the acidified slurry was bubbled at *p*H 2.0 to ensure the removal of any residual $^{14}CO_2$. The macromolecular and low-molecular-weight fractions were then separated by filtration (*3*), and the amount of ^{14}C incorporated into both organic fractions was determined by liquid scintillation counting (*4*). We determined the total unlabeled carbonate component of the pore water used in the incubations (4.49 mM CO_2 per liter) by bubble-stripping an acidified sample (*5*) and measuring the total CO_2 on an infrared analyzer (precision, ± 0.03 mM). The amount of CO_2 taken up from the unlabeled carbonate pool is shown as a profile in Fig. 1, and a maximum is evident at the 40-cm horizon. The problem of distinguishing between autotrophic and heterotrophic uptake of CO_2 (*6*) is not a major one at this horizon. Heterotrophic fixation of CO_2 requires energy from the oxidation of organic carbon and would not produce simultaneously the peaks of organic carbon and CO_2 fixation observed.

The chemoautotrophic sulfur bacteria (*7*) play at least a partial role in the uptake of CO_2, as shown in the results of additional incubations of sediment from 40 cm subbottom (Fig. 2). Uptake is enhanced by incubation with 1 mM sodium thiosulfate ($Na_2S_2O_3$), which indicates the presence of bacteria that are capable of oxidizing reduced forms of sulfur as an energy source for biosynthesis. In addition, the inhibition of CO_2 uptake in samples incubated under N_2 shows that O_2 is required, although possibly at low concentrations (*6*).

Our experiments were designed to simulate the natural microbial environment in that we allowed mixed bacterial populations to develop without applying the pure culture technique of media enrichment to favor the autotrophs. The experiments demonstrate that a microbial mechanism of organic carbon enrichment is present in the sediment examined. Jørgensen (*8*) has stated that many of the sulfur-oxidizing autotrophs

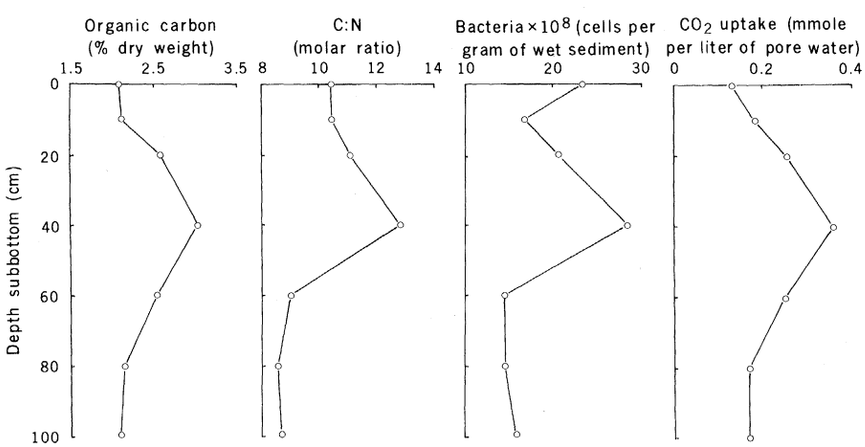

Fig. 1 (left). Distribution of organic carbon, the ratio of carbon to nitrogen, total bacterial cell counts, and CO_2 uptake with depth in a core from sulfide-rich marine muds.

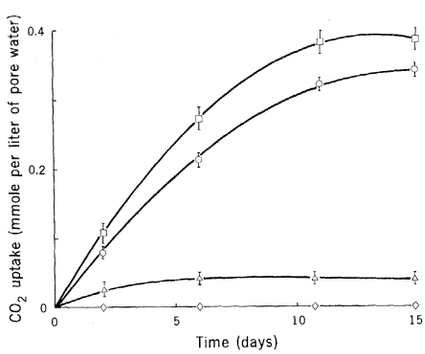

Fig. 2 (right). Uptake of CO_2 as a function of time in samples incubated aerobically without $Na_2S_2O_3$ (○), incubated aerobically with 1 mM $Na_2S_2O_3$ (□), incubated anaerobically (△), or killed and fixed with 3 percent gluteraldehyde at time zero and incubated aerobically (◇). Error bars delineate the standard deviation around the mean uptake values of triplicate incubations.

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