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# EFFECT OF OXYGEN TENSION ON THE GENERATION OF ALKANES AND MALONDIALDEHYDE BY PEROXIDIZING RAT LIVER MICROSOMES*

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**Abstract**—The alkanes, ethane and pentane, are often used as indices of lipid peroxidation. Because it has been indicated that $O_2$ tension can affect the yield of these compounds, a systematic study of this was carried out. Rat liver microsomes were peroxidized using an iron–ascorbate system. The incubations were carried out in sealed flasks at 37° under $N_2$ and various concentrations of $O_2$ up to 100%. Ethane and pentane production were measured by gas chromatography, and malondialdehyde was measured by the thiobarbituric acid reaction. Microsomal fatty acids were measured by gas chromatography. Polyunsaturated fatty acids were lost during lipid peroxidation. There was no loss of saturated or monounsaturated fatty acids. Loss of polyunsaturated fatty acids correlated with $O_2$ tension in the flask. Half-maximal losses of docosahexaenoic acid, arachidonic acid, and linoleic acid occurred at 3, 5, and 35% $O_2$ respectively. Malondialdehyde formation reflected polyunsaturated fatty acid loss at all $O_2$ concentrations. Alkane formation reflected polyunsaturated fatty acid loss below 5% $O_2$ but not above it. The ratio of alkane formed to precursor polyunsaturated fatty acid lost decreased progressively as $O_2$ concentration was increased above 5%. For example, the molar yield of pentane formed per precursor polyunsaturated fatty acid lost was 0.3% at 5% $O_2$ but only 0.003% at 100% $O_2$. This indicates that quantitation of lipid peroxidation using alkane formation requires consideration of $O_2$ tension at the site of alkane formation.

Current interest in lipid peroxidation as a pathogenetic process has led to a variety of approaches to its quantitation. Consumption of the substrates, polyunsaturated fatty acids and oxygen, can be measured in studies carried out *in vitro*. More commonly used, however, and required for *in vivo* studies, is measurement of products of lipid peroxidation such as malondialdehyde (MDA), conjugated dienes, fluorescent compounds, and the alkanes ethane and pentane [1]. Ethane and pentane arise from the peroxidative breakdown of $\omega$-3 and $\omega$-6 polyunsaturated fatty acids, respectively, and breath contents of these alkanes are commonly used as indices of lipid peroxidation *in vivo*.

Animals metabolize ethane and pentane [2], so breath content of these compounds can be altered by inhibitors or by damage to the liver [3], which appears to be the major organ responsible for alkane metabolism. This makes the use of these compounds as indices of lipid peroxidation more difficult.

Another possible problem of the hydrocarbon exhalation technique was pointed out by Cohen [4]. The precursors of the alkanes, the corresponding alkyl radicals, are formed by $\beta$-scission of alkoxy radicals on the side of the oxy radical group closest to the methyl end of the fatty acid [5]. These alkyl radicals can react by hydrogen abstraction yielding the alkanes but, alternatively, can react with oxygen to yield peroxy radicals. Further reaction of peroxy radicals could be the source of pentanol and ethanol, detected as products of peroxidizing $\omega$-6 and $\omega$-3 polyunsaturated fatty acids respectively [5]. With increasing $O_2$ tensions, the reaction with oxygen should become quantitatively more important, leading to lower yields of alkanes and distortion of lipid peroxidation estimates.

The present study was undertaken to evaluate the effect of $O_2$ tension on the commonly-used indices of lipid peroxidation: malondialdehyde, pentane, and ethane formation. The rat liver microsomal lipid peroxidation system was used in these experiments, and fatty acid loss was measured as the frame of reference.

## MATERIALS AND METHODS

Hepatic microsomes were isolated from male Sprague–Dawley rats (250–300 g body weight) by differential centrifugation [6]. The rats were fed Teklad pelleted rat diet purchased from Harlan Sprague–Dawley (Houston, TX). A buffer containing 50 mM Tris·HCl and 150 mM KCl was adjusted to pH 7.4 and used as the isolation and incubation medium. Incubations were carried out in sealed 25-ml flasks in a total volume of 5 ml and with a protein concentration of 1.4 to 1.6 mg/ml. The flask atmospheres with 10% oxygen or less were adjusted by flushing the flask with nitrogen for 15 min and injecting graded amounts of oxygen. All reagents used were purged with nitrogen.

Lipid peroxidation was initiated after a 5-min preincubation of microsomes at 37° by addition of 5 $\mu$M

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iron, 2 mM ADP, and 1 mM ascorbic acid [7]. When $CCl_4$ metabolism was used as an alternative radical source, $CCl_4$ was added as 5 μl of an ethanolic solution to a final concentration of 72 μM in the presence of 300 μM NADPH and 50 μM $Na_2$-EDTA [6].

After 10 min of incubation in a metabolic shaker, headspace samples from the flasks were analyzed for pentane and ethane by gas chromatography [3, 6]. Quantitation was achieved by assaying the atmosphere of buffer-containing flasks to which ethane and pentane had been added. These were used as standards. As an alternative index of lipid peroxidation, the formation of thiobarbituric acid (TBA)-reactive material, also referred to as malondialdehyde, was monitored [7].

Experiments were carried out to determine whether significant metabolism of hydrocarbons occurred in the incubation system. Pentane (0.31 nmole) was added to flasks containing microsomes, and the incubation was started by injection of NADPH to give a concentration of 300 μM. After 10 min, flask atmosphere was assayed for pentane. Controls with no NADPH added and with no pentane added were done. Incubations were carried out in duplicate. Flask atmospheres tested were 5% and 100% oxygen. Metabolism was assessed by comparing pentane concentration in the experimental flask with the sum of the concentrations in the controls. Under both oxygen concentrations, the apparent metabolism of pentane was less than 10%.

For fatty acid analysis 1-ml aliquots of the microsomal incubation mixture were extracted with 20 vol. of chloroform–methanol (3:2), containing 0.005% butylated hydroxytoluene as antioxidant after addition of margarinic acid and eicosadienoic acid as internal standards. The chloroform extract was evaporated to dryness in a nitrogen stream at room temperature. Saponification was carried out in methanolic KOH (1 M) at 70° for 15 min. Heating was continued for 5 min after addition of 7% boron trifluoride. After transfer to an ice bath, the fatty acid methyl esters were extracted into hexane and dried over anhydrous sodium sulfate.

Aliquots (1–2 μl) of this extract were used for gas chromatographic analysis. A Hewlett–Packard 5880A gas chromatograph (Hewlett–Packard, Avondale, PA), equipped with a flame ionization detector was used. Fatty acid methyl esters were separated on a 6-ft column packed with 10% SP 2330 (Supelco, Inc., Bellefonte, PA) with a temperature gradient from 100 to 200° at a programmed rate of 5°/min. Recoveries and individual fatty acid yields were calculated using the internal standards and fatty acid methyl ester reference mixtures. Peak area integrals were used for calculations.

NADPH and ascorbic acid were supplied by the Sigma Chemical Co., St. Louis, MO. The fatty acid methyl ester reference mixtures were purchased from Alltech Associates, Inc., Deerfield, IL.

## RESULTS

Rat liver microsomal membranes contain a large amount of unsaturated fatty acids. As shown in Table 1, the ω-6 unsaturated fatty acids, arachidonic acid and linoleic acid, are quantitatively the most important constituents of microsomal unsaturated phospholipids. The bulk of microsomal ω-3 unsaturated fatty acids was made up of docosahexaenoic acid. Only traces of linolenic acid were detectable. Under the dietary conditions of this study, ω-3 and ω-6 unsaturated fatty acids were detected in microsomal membranes in a molar ratio of 0.15. Incubation at 37° for 10 min without a free radical-generating system did not lead to loss of unsaturated fatty acids (not shown).

Initiation of peroxidative reactions in the presence of the iron · ADP complex and ascorbate resulted in a striking change of microsomal fatty acid composition. In Fig. 1, microsomal fatty acid loss is shown as a function of oxygen tension. Individual fatty acid contents were normalized with respect to stearic acid, which as a saturated fatty acid was not subject to peroxidative destruction and, therefore, was used

Table 1. Rat liver microsomal fatty acid composition

| Fatty acid | | Concentration (nmoles/mg protein) |
|---|---|---|
| Palmitic acid | (16:0) | 348 ± 9.3 |
| Palmitoleic acid | (16:1) | <1 |
| Stearic acid | (18:0) | 488 ± 5.8 |
| Oleic acid | (18:1) | 122 ± 8.1 |
| Linoleic acid | (18:2) | 260 ± 6.5 |
| Linolenic acid | (18:3) | 2.4 ± 0.9 |
| Arachidonic acid | (20:4) | 404 ± 9.5 |
| Docosahexaenoic acid | (22:6) | 94.2 ± 10.8 |

Values, except for palmitoleic acid, are means ± SD, N = 4.

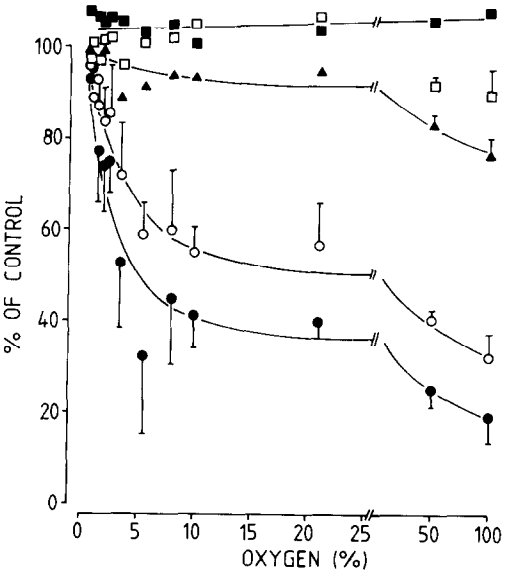


Fig. 1. Oxygen dependence of microsomal fatty acid loss during iron · ADP-induced lipid peroxidation. Individual fatty acid contents, docosahexaenoic (●), arachidonic (○), linoleic (▲), oleic (□) and palmitic (■) acid, are expressed relative to microsomal stearic acid concentration. The 100% values were obtained from non-peroxidized microsomes as given in Table 1. Values are means ± SD, N = 4.

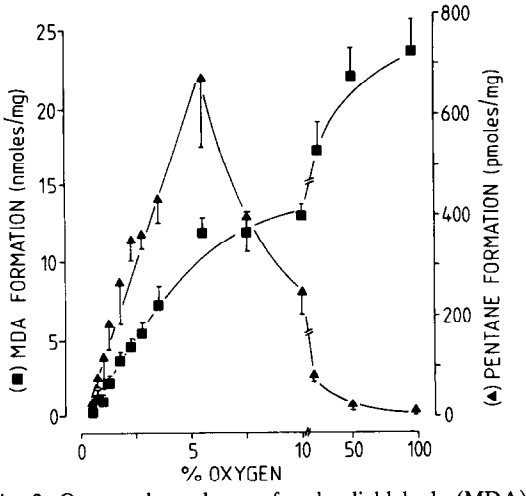


Fig. 2. Oxygen dependence of malondialdehyde (MDA) (■) and pentane (▲) formation in the incubations described in Fig. 1.

to correct for differences in extraction efficiency. Only unsaturated fatty acids with at least two double bonds were peroxidized as the oxygen tension in the incubation atmosphere increased. The susceptibility to peroxidative fatty acid loss decreased in the order docosahexaenoic acid > arachidonic acid > linoleic acid as judged from a maximal loss of 82, 67 and 23%, respectively, in the presence of 100% oxygen.

The sensitivity of unsaturated fatty acid to peroxidative reactions under low oxygen tensions followed the same trend. Half-maximal levels of docosahexaenoic acid, arachidonic acid and linoleic acid loss were reached in the presence of 3, 5, and 35% oxygen, respectively.

In Fig. 2 malondialdehyde and pentane formation by these same peroxidizing microsomal suspensions is shown. While malondialdehyde formation correlated with unsaturated fatty acid loss under all oxygen tensions studied, pentane showed a biphasic oxygen dependence. Pentane and malondialdehyde formation both extrapolate to zero under anaerobic conditions. Below 5% oxygen, a close relationship existed between the rate of pentane or malondialdehyde formation on the one hand and oxygen tension on the other. However, a further increase in oxygen tension decreased pentane formation exponentially while increasing malondialdehyde formation. Pentane formation had declined to half-maximum at 7.5% oxygen. Experiments were performed to assess metabolism of pentane by microsomes with NADPH added. Even under 100% oxygen there was less than 10% apparent metabolism of pentane indicating that the oxygen effect was on formation and not on metabolism.

Ethane and pentane were formed under all oxygen tensions studied at a constant ratio of 0.19 ($r = 0.955$). Therefore, the effect of oxygen is similar for both alkanes.

In Table 2, the loss of unsaturated fatty acids as well as malondialdehyde and alkane formation under different oxygen tensions is compiled. One mole of malondialdehyde was formed on loss of 14–21 moles of unsaturated fatty acid for a molar yield of approximately 5%. Oxygen tension had no systematic effect on malondialdehyde yield. Below 5% oxygen, pentane was formed from $\omega$-6 unsaturated fatty acids, i.e. arachidonic acid and linoleic acid, at a molar yield of approximately 0.3%. This yield dropped to 0.05% at 21% oxygen and 0.003% at 100% oxygen. Ethane formation had a similar oxygen dependence to that of pentane formation.

The oxygen dependence of the ratio of malondialdehyde to pentane is shown in Fig. 3. At oxygen tensions below 5%, in the presence of the iron-ascorbate system, approximately 12 moles of malondialdehyde were formed per mole of pentane. As the oxygen tension rose above 5%, the molar ratio of malondialdehyde to pentane increased in a linear fashion. However, this ratio of malondialdehyde to hydrocarbon was dependent on the radical source. When $CCl_4$-derived radicals initiated the peroxidative process (see insert in Fig. 3), the yield of malondialdehyde relative to pentane was significantly higher at 5% oxygen and below than when the iron-ascorbate system was used.

These results show that malondialdehyde formation coincided with the loss of polyunsaturated fatty acids in peroxidizing microsomes under all oxygen tensions used. In contrast, hydrocarbon yield was a complex function of oxygen tension. Hydrocarbons were produced in quantities comparable to malondialdehyde under nearly anaerobic conditions, but hydrocarbon formation during peroxidation dropped to less than 1% compared with malondialdehyde formation when oxygen tension was increased from 5 to 100%.

## DISCUSSION

In the present study, the oxygen dependence of microsomal lipid peroxidation was investigated. Formation of malondialdehyde and alkanes, two peroxidation products, and loss of microsomal fatty acid, a substrate of lipid peroxidation, were measured.

The differential susceptibility of polyunsaturated fatty acids as well as the apparent inertness of monounsaturated fatty acids to peroxidation (Fig. 1) is in agreement with studies by other laboratories. The yield of 1 mole malondialdehyde on loss of approximately 17 moles of unsaturated fatty acids agrees with yields reported by May and McCay [8] and Baker and Wilson [9]. The extent of individual fatty acid loss and stimulation of the peroxidation rate under low oxygen tensions increased with the number of double bonds in the unsaturated fatty acid. This is compatible with decreasing rate constants of the reaction of the initiating/propagating free radicals with unsaturated fatty acids in the order docosahexaenoic acid > arachidonic acid > linoleic acid which contain 6, 4 and 2 double bonds respectively. A similar relationship was shown by Forni *et al.* [10] for the reaction of the $CCl_3OO\cdot$ radical with purified unsaturated fatty acids using pulse radiolysis. The deviation of the ratio of ethane to pentane formation (0.19) from the ratio of $\omega$-3 to $\omega$-6 unsaturated fatty acid content of microsomal membranes (0.15) also reflects the increased sus-

Table 2. Oxygen dependence of lipid peroxidation

| Oxygen tension | Fatty acid loss (nmoles/mg · 10 min) Docosahexaenoic acid | Arachidonic acid | Linoleic acid | Oleic acid | Malondialdehyde (nmoles/mg · 10 min) | Pentane |
|---|---|---|---|---|---|---|
| 1% | 10.5 ± 17 | 40.4 ± 74 | 2.8 ± 13 | 5.7 ± 15 | 0.99 ± 0.74* | 0.124 ± 0.046* |
| 5% | 65.4 ± 25* | 165 ± 25* | 22.0 ± 11* | 6.4 ± 14 | 11.9 ± 0.9* | 0.696 ± 0.141* |
| 21% | 59.1 ± 13* | 172 ± 58* | 12.2 ± 12 | 3.0 ± 30 | 17.5 ± 1.9* | 0.085 ± 0.008* |
| 100% | 77.7 ± 14* | 273 ± 36* | 58.8 ± 7.4* | 17.8 ± 17 | 23.8 ± 1.6* | 0.010 ± 0.004* |

Values are means ± SD, N = 4.

* Significantly different from non-peroxidized value by Student's *t*-test, $P < 0.05$. See Table 1 for fatty acid non-peroxidized values; MDA and pentane values were 0 in non-peroxidized microsomes.

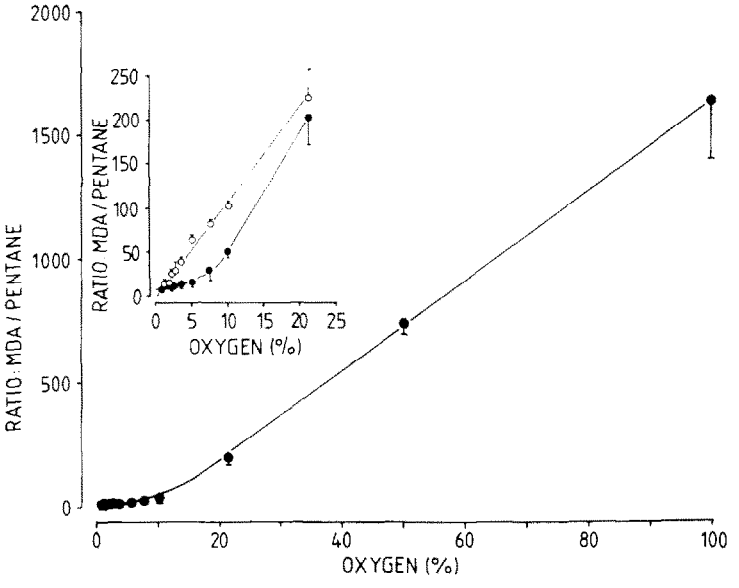


Fig. 3. Oxygen dependence of the ratio of malondialdehyde (MDA) to pentane formation by microsomes peroxidized in the presence of the iron · ADP complex and ascorbate (●) or $CCl_4$ and NADPH (○). Values are means ± SD, N = 4.

ceptibility to peroxidative processes of docosahexaenoic acid compared to arachidonic acid and linoleic acid.

In the iron · ADP system, malondialdehyde formation was proportional to the loss of unsaturated fatty acids under all oxygen tensions. The biphasic oxygen effect on alkane formation indicates the presence of two types of oxygen-dependent reactions affecting alkane formation. At low oxygen tensions, radical formation to initiate and/or maintain free radical reactions is limiting. With increasing oxygen tensions interference with alkane formation becomes evident. Two possibilities seem conceivable to explain this inhibitory effect of oxygen: (a) At higher oxygen tensions the proportion of oxygen-containing radicals in the propagation of free radical chain reactions increases and may favor the formation of endoperoxides, which are less susceptible to β-scission. Thereby, the release of hydrocarbons may be decreased without affecting the formation of TBA-reactive material, which is released under the more drastic conditions of the TBA assay. (b) Alkyl radicals, once formed, can undergo oxygen-dependent reactions. Free radicals react either by hydrogen abstraction, resulting in chain propagation and alkane evolution, or by recombination with other radicals. Ground state molecular oxygen *per se* is a diradical and its high solubility in water makes it a major radical species in incubations when oxygen tensions are increased. For example, aerated water contains 240 μM oxygen, which is probably orders of magnitude higher than the concentration of any other radical produced during microsomal lipid peroxidation. Recombination reactions with molecular oxygen compared to hydrogen abstraction are likely to increase with rising oxygen tensions and result in an oxygen concentration-dependent inhibition of alkane formation.

The relative importance of different oxygen-dependent free-radical reactions is also influenced by the nature of the radical-generating system. Initiation of lipid peroxidation by $CCl_4$-derived radicals and by the iron–ascorbate system differs in the nature and the quantity of the initiating radicals, as well as their dependence on oxygen. The iron–ascorbate system is a representative of oxygen-dependent radical-generating systems, whereas $CCl_3\cdot$ radicals are formed by reductive dehalogenation, a process which is inhibited progressively by oxygen. As a result, potentiation of lipid peroxidation product formation *in vivo* or *in vitro* is observed under hyperoxic conditions by a variety of redox cyclers or the iron–ADP system, while radical chain reactions initiated by reductive activation of compounds such as $CCl_4$ are facilitated by hypoxia. The hydrocarbon yields in the above-mentioned systems differ under low oxygen tensions, although the yields in both systems are comparable at higher oxygen tensions. The biphasic oxygen dependence of the malondialdehyde versus pentane ratio observed in the presence of the iron–ADP complex may suggest that most of the oxygen consumed at oxygen tensions below 5% was activated by the iron–ADP complex while interference with alkane formation by oxygen remained of only minor importance. Thus, a nearly constant ratio of pentane to malondialdehyde was observed under these conditions. With $CCl_4$-metabolizing microsomes, this biphasic oxygen dependence was not found. Low metabolic activation rates (max. 1–2 nmoles/mg·min) may have shifted the threshold oxygen tension below which oxygen limits the initiation and propagation of free-radical reactions to a level too low for its detection in these experiments.

The differential oxygen dependence of reactions associated with lipid peroxidation has two implications for biological systems. First, the different susceptibility of polyunsaturated fatty acids toward lipid peroxidation under low oxygen tensions will result in a preferential loss of docosahexaenoic acid over less unsaturated fatty acids in the presence of prooxidants. As unsaturated fatty acids are not distributed uniformly among different phospholipid classes, loss of docosahexaenoic acid may affect mainly certain phospholipid types, causing a disproportionate effect on certain membrane functions.

Second, the oxygen dependence of alkane formation during peroxidation *in vitro* as shown in this study is likely to reflect similar processes in intact cells and organisms. Certainly radical generation rates and local oxygen supply will affect the steady-state oxygen tension in cells and cell organelles and therefore influence the hydrocarbon yield. Even though metabolism of hydrocarbons was minimal in the system used in these studies, it occurs *in vivo* [2] and must be considered in evaluating exhaled alkanes. Effects of the normal range of tissue oxygen tensions on alkane metabolism need to be evaluated as an additional factor in affecting exhalation of alkanes.

Because of these considerations, it seems likely that attempts to calculate absolute peroxidation rates *in vivo* on the basis of hydrocarbon yields determined in model systems will give erroneous results. Hydrocarbon release can be expected to be a much more sensitive index of lipid peroxidation in tissues or cell populations exposed to low oxygen tensions than in tissues with higher oxygen tensions. Indeed alkanes have been used so far mainly as a probe of lipid peroxidation in injury of the centrilobular region of the liver, the location where the lowest hepatic oxygen tensions are encountered. Application of the hydrocarbon exhalation technique to study peroxidative processes *in vivo* under different oxygen tensions is compromised by differences in alkane yield, which may lead to an over- or underestimation of actual peroxidative processes. Increase in oxygen tension from the range of 5 to 100% reduces the efficiency of hydrocarbon formation to approximately 1%, which diminishes the value of this technique in the study of peroxidative processes in better oxygenated tissues such as the lung *in vivo*, cell cultures, and *in vitro* incubations under air or oxygen.

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