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Formation of pentane versus 1-pentanol in the ferrous sulfate-initiated decomposition of 15-hydroperoxyeicosatetraenoic acid in hypoxic and hyperoxic conditions

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Efforts to examine the contributions of lipid peroxidation to biological processes are confronted with the formidable problem of adequate measurement of the nature and extent of peroxidation of cellular lipid [1]. One index of lipid peroxidation that has received considerable attention over the last several years is the measurement of the hydrocarbons ethane and pentane. The measurement of these hydrocarbons offers superb sensitivity, and the noninvasive nature of these measurements permits repeated determinations from an individual sample, live animal, or human subject. However, the extent to which ethane and pentane expiration will reflect the extent of lipid peroxidation will be affected by several factors, and these factors must be considered in interpreting data derived from measurement of expired hydrocarbons.

One of these factors is the concentration of oxygen. A number of studies of lipid peroxidation in biological systems have been reported in which an inverse or biphasic relationship between oxygen tension and ethane or pentane formation was observed [2–7]. Much of the effect of increased oxygen concentrations on CCl_4-induced production of ethane and pentane has been attributed to inhibition by O_2 of the reductive metabolism of CCl_4 to produce $^{\cdot}CCl_3$ and $^{\cdot}OOCCl_3$, the metabolites responsible for initiation of lipid peroxidation [2, 3]. Hepatic oxygen status also might affect net pentane expiration if oxygen availability became limiting for metabolism of pentane, but a similar effect on ethane measurements would not be expected, inasmuch as ethane is not metabolized significantly [6]. In addition, tissue oxygen concentrations might influence alkane production through possible effects on the availability and redox status of iron chelates. However, the present data demonstrate that dramatic changes in alkane yield caused by changes in oxygen concentration can be produced through trapping the alkyl radical by molecular oxygen, evidenced in the present study by subsequent transformation of the pentyl fragment to the respective primary alcohol.

Materials and Methods

Reagents were purchased from the Sigma Chemical Co. (St Louis, MO) and used without further purification. Arachidonic acid was oxidized with soybean lipoxygenase and 15-HPETE* purified as described [8]. Buffer was degassed through four freeze–thaw cycles and maintained under an atmosphere of nitrogen for the low-oxygen buffer. Alternatively, oxygen-saturated buffer was prepared by bubbling degassed buffer with pure oxygen. 15-HPETE (600 nmol) was introduced to glass tubes capped with gastight, Teflon-lined rubber septa containing 2 ml of buffer under the appropriate N_2 or O_2 atmospheres. Decomposition of the 15-HPETE was initiated by injection of excess $FeSO_4$ in buffer. Pentane production was measured by gas chromatographic analysis of the headspace. A 2 m × 2 mm i.d. glass column packed with activated alumina was employed with flame ionization detection. Hexanal and the pentanols were measured by extraction of the aqueous phase with 100 μl of dichloromethane and gas chromatographic analysis on a 2 m × 2 mm i.d. glass column packed with Carbowax 20M. Identity was determined by coelution with authentic standards, and quantitation was obtained by comparison of chromatographic peak areas with areas produced by injection of known amounts of standards, corrected for extraction efficiency.

Statistical analysis of the data employed the unpaired Student's *t*-test [9].

Results and Discussion

In the system employed in the present studies, the addition of $FeSO_4$ to 15-HPETE decomposed greater than 99% of the hydroperoxide and produced pentane in a 2.5% yield (Table 1). Oxygen saturation of the buffer and atmosphere inhibited pentane production by more than 99%. The formation of 1-pentanol (**9**) was enhanced markedly in the oxygenated system, presumably due to trapping of the 1-pentyl radical (**4**) before it can be converted to pentane (**8**) via hydrogen atom abstraction (Fig. 1). Neither 2-pentanol nor 3-pentanol was detected. Because 2- and 3-pentanol are the initial products of pentane metabolism [10], the relative contributions of radical trapping by O_2 versus mixed-function oxidase metabolism of pentane in biological systems should be definable by these methods. The small amount of 1-pentanol formed in the deoxygenated system (Table 1) probably reflects the incomplete deoxygenation of the system.

The fact that buffer saturation with oxygen resulted in an increase in 1-pentanol formation that was larger than the decrease in pentane formation probably reflects the effectiveness of the competition by molecular oxygen for the pentyl radical versus fates of the pentyl radical other than hydrogen atom abstraction (e.g. radical addition to carbon—carbon double bonds or radical combination reactions). The increased yield of 1-hexanal (**6**) under oxygenated conditions may, in part, be a result of inhibition of processes involving alkyl radical attack on hexanal but the nature of this effect remains to be elucidated. Interestingly, Vaz and Coon [11] have reported that the anaerobic decomposition of an analogous omega-6 hydroperoxide (13-hydroperoxy-9,11-octadecadienoic acid) by a reconstituted cytochrome P-450 system gives

Table 1. Effect of oxygen on products of iron-catalyzed decomposition of 15-HPETE

	Pentane	1-Pentanol (% yield)	1-Hexanal
N_2	2.52 ± 0.02	0.54 ± 0.04	15.85 ± 1.95
O_2	0.01 ± 0.01*	8.48 ± 0.81*	26.25 ± 1.05*

15-HPETE (600 nmol) was placed in glass tubes containing 2 ml KPO_4 buffer, pH 6.8, saturated either with N_2 or with O_2. Excess $FeSO_4$ was added, and pentane was measured by gas chromatographic analysis of headspace samples. The aqueous phase was extracted with 100 μl of methylene chloride, which was analyzed by GC for pentanols and hexanal. Data are means ± SEM for three determinations.

* The product yields in the presence of oxygen are statistically different from those observed in the nitrogen-saturated samples ($P < 0.05$).

* Abbreviations: 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; and LOH, lipid hydroxy acid.

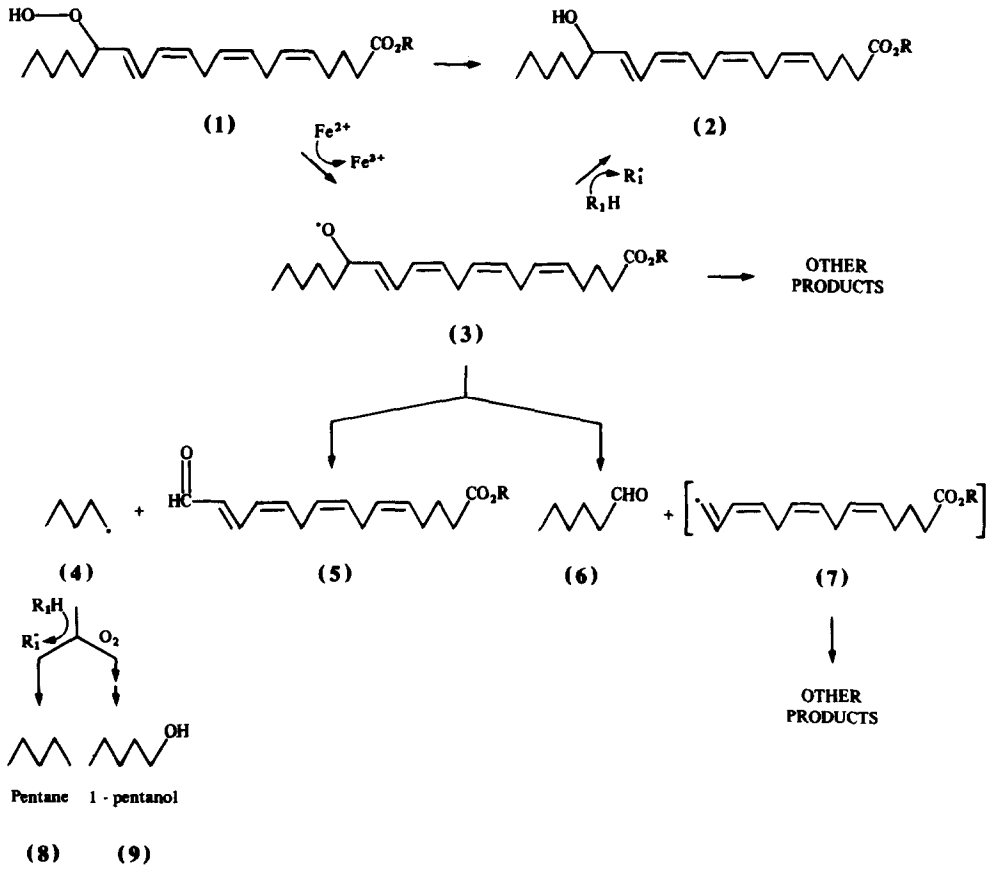

Fig. 1. A mechanistic explanation for the effects of oxygenation on the production of pentane versus 1-pentanol in the iron-induced decomposition of 15-HPETE.

pentane in greater than 80% yield, which suggests that the free alkoxyl radical analogous to (3) is not an intermediate in the cytochrome P-450 mediated reaction and that a different mechanism may be involved in that system.

The present data demonstrate that disproportionate increases in alkane expiration could arise from hypoxia as well as from an actual increase in lipid peroxidation. In our recent study of diquat-induced alkane expiration [12], we were concerned about the possibility of hepatic anoxia arising from the consumption of oxygen during diquat redox cycling and, therefore, measured hepatic ATP content. Administration of diquat did not alter hepatic ATP content, which decreases rapidly during hypoxia, indicating that tissue hypoxia was not responsible for the observed increases in ethane and pentane expiration.

In summary, ethane and pentane expiration are highly sensitive and potentially useful indices of processes involved in the formation and disposition of lipid hydroperoxides *in vitro* or *in vivo*. However, the complicating features of reduction [e.g. (1) → (2)] versus β-scission [(1) → (4–7)] make it essential to evaluate tissue LOH content versus alkane expiration. The highly effective trapping of the 1-pentyl radical by oxygen, demonstrated in the present report, suggests that 1-pentanol and similar products also may serve as useful indices of peroxidation and β-scission processes, particularly in the pathogenesis of hyperoxia where alkyl radical trapping by molecular oxygen may become dominant. However, additional investigations are needed to determine the validity of this speculation.

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Fluorometric assay of hepatic microsomal monooxygenases by use of 7-methoxyquinoline*

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Cytochrome P-450 monooxygenases constitute one of the most potent systems to deal with the metabolism of foreign substances. The hepatic microsomal monooxygenase system usually metabolizes nonpolar xenobiotics to more polar compounds. The monooxygenase system operating in hepatic microsomes is comprised of NADPH–cytochrome P-450 reductase and cytochrome P-450, flavo- and hemoproteins respectively. The central catalytic unit, cytochrome P-450, is subject to induction by a variety of chemicals which stimulate the biosynthesis of different forms of the cytochrome. Extensive reviews on the subject of chemical induction of cytochromes P-450 are available [1–3].

Many assays have been developed to determine the activities of the constitutive phenobarbitol (PB) and 3-methylcholanthrene (MC)-induced forms of the cytochrome P-450 monooxygenases. Only a few of these are direct assays, i.e. assays where enzymatic activity can be determined immediately. Fluorometric assays have been commonly utilized for the direct measurement of monooxygenases because of their sensitivity and the ability to select fluorophores that fluoresce in a wavelength range that obviates artifacts resulting from endogenous materials or other reaction components. Direct fluorescent assays include the O-dealkylation of coumarins (umbelliferones) [4–6], the O-dealkylation of phenoxazones (resorufins) [7–9] and the O-dealkylation of fluorescein [10].

While all of the above-mentioned substrates measure monooxygenase activity, two have been demonstrated to be specifically metabolized by the PB-induced (7-pentoxyresorufin) [9, 11] and MC-induced (7-ethoxyresorufin) [6, 8, 11, 12] monooxygenases. The reason for the high degree of metabolic specificity with the resorufin substrates is not immediately apparent. However, it is true that of the available fluorescent substrates the resorufins are the only ones that contain both oxygen and nitrogen atoms in the fluorophore ring system; the others contain oxygen atoms only. It would, therefore, be of interest to know if the presence of a nitrogen in the fluorophore ring system allows the specific metabolism of the substrate by certain cytochrome P-450 monooxygenases.

We have been investigating a series of 7-alkoxyquinolines to determine the effect of the nitrogen atom in the aromatic ring system on metabolic specificity. This report establishes 7-methoxyquinoline as a substrate for the assay of hepatic microsomal cytochrome P-450 monooxygenases. Synthesis, fluorescence characteristics, and comparison of metabolic activities with hepatic microsomal preparations from rats previously treated with MC and PB are presented.

Materials and Methods

Chemicals. NADPH, phenobarbital and Tris were from the Sigma Chemical Co. (St. Louis, MO). 3-Methylcholanthrene was purchased from Fluka AG (Buchs, Switzerland). 7-Quinolinol and iodomethane were obtained from Kodak Laboratory Chemicals (Rochester, NY).

Animals and sample preparation. Animals were obtained from a reproducing colony of Wistar rats at the Institute for Pharmacology and Toxicology, Marburg, FRG. Male rats of approximately 200 g were used in the experiments. Animals were induced with PB (0.1% in drinking water for 6 days) and MC (two i.p. injections of 30 mg/kg body weight, in peanut oil). Controls consisted of animals injected twice with 0.5 to 1.0 ml of peanut oil only. The animals were killed by cervical dislocation 2 days after the last 3-MC treatment. Hepatic tissues (two livers for each treatment group of rats) were homogenized in 4 vol. of 20 mM Tris–HCl (pH 7.6) containing 150 mM KCl and 1 mM EDTA. The homogenates were centrifuged at 1000 *g* for 10 min and then the supernatant fractions were removed and centrifuged at 10,000 *g* for 10 min. The microsomal pellets were obtained by centrifuging the 10,000 *g* supernatant fractions at 100,000 *g* for 60 min, resuspending the resulting pellets in 20 mM Tris–HCl (pH 7.6, 150 mM KCl, 3 mM $MgCl_2$), and recentrifuging at 100,000 *g* for another 60 min. The microsomal pellets were finally suspended (1 ml resuspension buffer per g fresh liver weight) in 20 mM Tris–HCl (pH 7.6) containing 150 mM KCl, 3 mM $MgCl_2$ and 15% glycerine.

Cytochrome P-450 concentrations were determined by

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