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Pentane as an Index of *in Vitro* Lipid Peroxidation *via* Microsomal NADPH-P-450 Enzyme Systems

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ABSTRACT

Pentane was measured in a mixture of guinea pig liver microsomes and carbontetrachloride (CCl_4) in the presence of nicotineamide adenine dinucleotide phosphate (NADPH) and under anaerobic conditions by gas chromatography. Pentane of 0.2 pmol/mg protein/min was formed in the presence of NADPH without CCl_4. Pentane formation increased to 2.0 pmol/mg protein/min in the presence of CCl_4 and NADPH. This reaction required an anaerobic atmosphere, 2.1 mmol/litter of NADPH and 13.4 mmol/litter of CCl_4 under optimal conditions. The formation was reduced in the presence of oxygen, glutathione, vitamin E and metyrapone. These results clearly show that pentane is formed by lipid peroxidation initiated by the free radical cleavage products of CCl_4, which are anaerobically produced by NADPH-dependent microsomal enzymes. We conclude that pentane is a good index of *in vitro* lipid peroxidation *via* microsomal NADPH-P-450 enzyme systems.

Key words: *Lipid peroxides, Pentanes, Microsome, Liver, Carbontetrachloride*

Hydrocarbon gases are known to be products of lipid peroxidation[4]. Many studies *in vivo* have employed pentane or ethane measurements of expired gas as an index of lipid peroxidation. However, studies of *in vitro* lipid peroxidation have commonly used other methods, such as the measurement of diene conjugation, malondialdehyde and fluorescent products. The measurement of pentane has not been as popular.

The purpose of this study is to determine the optimal conditions for pentane formation by carbontetrachloride (CCl_4) in guinea pig liver microsomes and to examine whether pentane formation can be used as an index of lipid peroxidation caused by microsomal enzyme systems.

MATERIALS AND METHODS

1) Materials

CCl_4 was obtained from Kanto Chem (Japan). Nicotineamide adenine dinucleotide phosphate (NADPH) was purchased from Boehringer Mannheim (West Germany). All other reagents were of analytical grade. Authentic pentane and ethane were used to determine the retention time.

2) Animals

Adult male guinea pigs weighing 225g to 275g were used in the experiments. After a 24-hour fast, the guinea pigs were killed by a blow to the head. The livers were immediately excised. After irrigation with cold physiologic saline solution through the portal vein to remove blood, the livers were homogenized in 0.05M potassium phosphate buffer pH 7.4. The homogenates were centrifuged at 8,000 $\times$ *g* for 10 min and the supernatants were further centrifuged at 105,000 $\times$ *g* for 1 hr. The microsome fraction was washed in 0.05 M potassium phosphate buffer, pH 7.4 by centrifugation at 105,000 $\times$ *g* for 1 hr. The microsomal fraction was then suspended in 0.1 M potassium phosphate buffer, pH 7.4.

3) Incubation systems

Under anaerobic conditions, nitrogen was sealed in a 12.3ml silicon-capped test tube containing the microsomal suspension (final volume of 3ml, pH 7.4, 0.1 M potassium phosphate buffer). After the addition of NADPH (2.1 mmol/litter) and CCl_4, the reaction was carried out at 37°C, for 15 min. After the reaction, 0.5 ml of the gas phase was analyzed by gas chromatography.

4) Quantitative analysis of pentane

The gas chromatograph used in this study was a Shimadzu GC-4B model equipped with a flame ionization detector. The liquid phase was dioctylphthalate packed in a 5m $\times$ 4mm glass column.

RESULTS

1) Analysis of pentane

Fig. 1 shows a gas chromatogram of head space gas from the incubation system without CCl_4 (A) and with CCl_4 (B). Retention time of pentane was 2.0 min. Ethane was not separated from air peak which was observed at 0.2 min with this column.

2) Effect of varying oxygen concentrations on pentane formation.

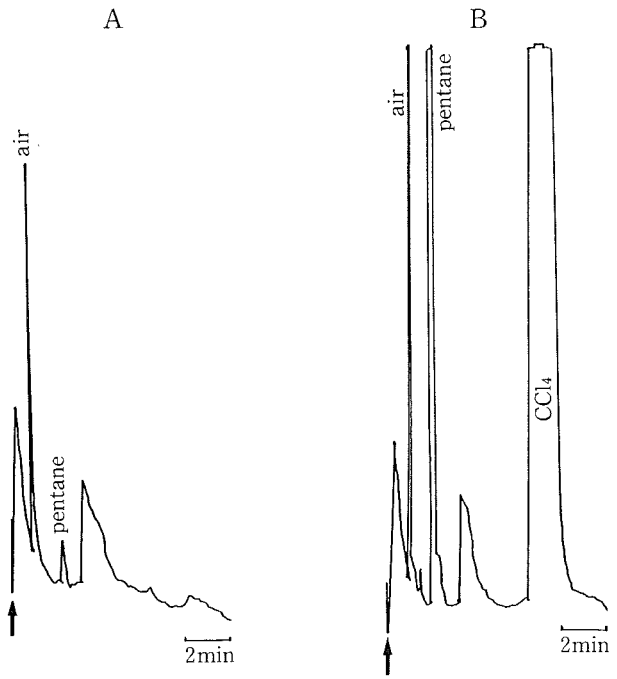

Fig. 1. Gas chromatogram of head space gas from the incubation system without CCl_4 (A) and with CCl_4 (B). The incubation system consisted of a microsomal suspension (total volume of 3 ml, pH 7.4, 0.1 M potassium phosphate buffer), NADPH (2.1 mmol/litter) without CCl_4, with CCl_4 (13.8 mmol/litter). The reaction was carried out at 37°C, for 15 min. Retention time of pentane was 2.0 min.

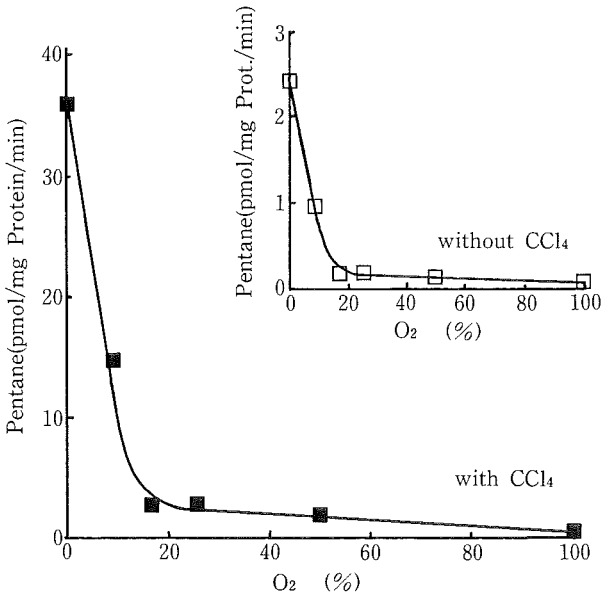

Fig. 2. Effect of varying concentrations of oxygen in the gas phase on pentane formation. The incubation system consisted of a microsomal suspension (total volume of 3 ml, pH 7.4, 0.1 M potassium phosphate buffer), NADPH (2.1 mmol/litter) with CCl_4 (13.8 mmol/litter), without CCl_4.

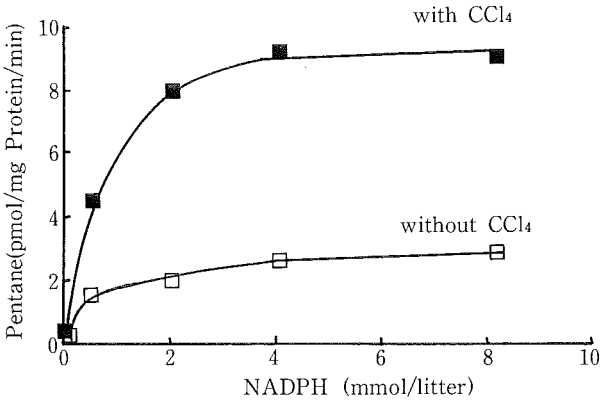

Fig. 3. Effect of varying concentrations of NADPH on pentane formation under anaerobic conditions. The incubation system consisted of microsomal suspension (total volume of 3 ml, pH 7.4, 0.1 M potassium phosphate buffer), NADPH (0.5, 2.1, 4.1, 8.2 mmol/litter) with CCl_4 (13.8 mmol/litter) without CCl_4.

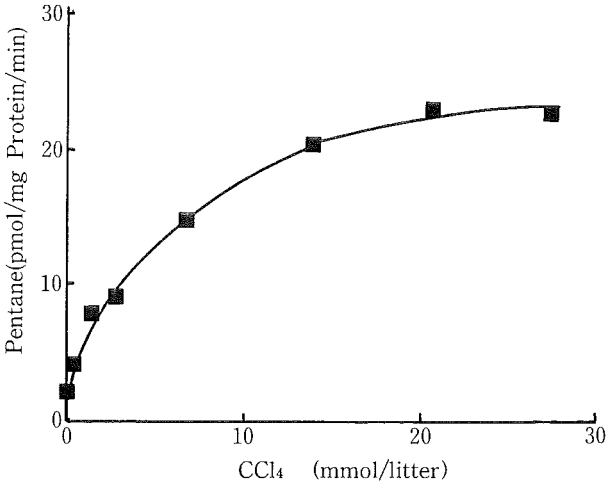

Fig. 4. Dose-response relation between pentane formation and CCl_4 under anaerobic conditions. CCl_4 (0—334 mmol/litter) was added to the incubation system. The incubation system consisted of a microsomal suspension (total volume of 3 ml pH 7.4, 0.1 M potassium phosphate buffer), NADPH (2.1 mmol/litter).

Fig. 2 demonstrates that pentane formation depends on the concentration of oxygen in the incubation mixture. In the presence of CCl_4, pentane of 36.0 pmol/mg protein/min was formed in anaerobic condition. Pentane formation decreased alongside increasing oxygen concentrations and reached 0.1 pmol/mg protein/min under 100% oxygen.

3) Effect of varying NADPH concentrations on pentane formation.

The formation of pentane increased in response to NADPH concentrations and reached a plateau (9.3 pmol/mg protein/min) in the presence of 2.1 mmol/litter NADPH. There was no formation of pentane without NADPH. Pentane formation without CCl_4 was also a response to NADPH (Fig. 3).

4) Dose-response relation between pentane forma-

Table 1. Relation between pentane formation and protein concentration of the microsomal suspension under anaerobic conditions[a].

Protein Concentration (mg/ml)	without CCl_4 (pmol/min)	with CCl_4 (pmol/min)
1.60	0.79	82.2
0.82	0.38	51.4
0.35	0.31	19.7
0.13	0.13	10.6
0.05	0.04	5.4

[a]: The incubation system consisted of a microsomal suspension (total volume of 3 ml, pH 7.4, 0.1 M potassium phosphate buffer), NADPH (2.1 mmol/litter), with CCl_4 (13.8 mmol/litter) or without CCl_4. Calculated regression line y = 50.3x + 4.2 with a correlation coefficient r = 0.99 with CCl_4; y = 0.5x + 0.1, r = 0.98 without CCl_4.

Table 2. Effect of Vitamin E on pentane formation under anaerobic conditions[a].

Vitamin E (nmol/litter)	without CCl_4 (pmol/mg protein/min)	with CCl_4 (pmol/mg protein/min)
0.0	1.5	23.7
4.5	1.4	22.8
11.3	0.3	18.7
22.5	0.1	17.1
45.0	0.1	12.2

[a]: Vitamin E (4.5, 11.3, 22.5, 45.0 nmol/litter) was added to the incubation system. The incubation system consisted of a pH 7.4, 0.1 M potassium phosphate buffer), NADPH (2.1 mmol/litter), with CCl_4 (13.8 mmol/litter) or without CCl_4.

Table 3. Effect of GSH on pentane formation under anaerobic conditions[a].

GSH (umol/litter)	without CCl_4 (pmol/mg protein/min)	with CCl_4 (pmol/mg protein/min)
0.0	0.4	23.6
1.0	0.3	11.9
5.0	0.2	6.6
10.0	0.1	5.4
20.0	0.1	5.2

[a]: GSH (0, 1.0, 5.0, 10.0, 20.0 umol/litter) was added to the incubation system. The incubation system consisted of a microsomal suspension (total volume of 3 ml, pH 7.4, 0.1 M potassium phosphate buffer), NADPH (2.1 mmol/litter), with CCl_4 or without CCl_4.

tion and CCl_4 administration.

Fig. 4 shows that pentane formation increased with increasing CCl_4 concentrations. A small amount of pentane (0.2 pmol/mg protein/min) was produced without CCl_4. Pentane formation increased to 2.0 pmol/mg protein/min in the presence of 13.4 mmol/litter of CCl_4.

5) Relation between pentane formation and protein concentration of the microsomal suspension.

Table 4. Effect of metyrapone on pentane formation under anaerobic conditions[a].

Metyrapone (mmol/litter)	without CCl_4 (pmol/mg protein/min)	with CCl_4 (pmol/mg protein/min)
0.0	0.3	14.7
0.5	0.2	11.7
10.0	0.1	9.3
15.0	0.1	7.3

[a]: Metyrapone (5, 10, 15 mmol/litter) was added to the incubation system. The incubation system consisted of a microsomal suspension (total volume of 3 ml, pH 7.4, 0.1 M potassium phosphate buffer), NADPH (2.1 mmol/litter), with CCl_4 or without CCl_4.

Pentane formation with or without CCl_4 showed a linear response to the protein concentration (r = 0.99 with CCl_4 and 0.98 without CCl_4) of the microsomal suspension (Table 1).

6) Effect of varying concentrations of vitamin E and glutathione (GSH) on pentane formation.

Both Vitamin E (Table 2) and GSH (Table 3) reduced pentane formation in the presence or absence of CCl_4. The addition of Vitamin E of 45 nmol/litter caused a 49% reduction in pentane production with CCl_4. GSH of 20 umol/litter caused a 78% decrease of pentane formation with CCl_4.

7) Effect of varying metyrapone concentrations on pentane formation (Table 4).

Metyrapone also reduced pentane formation in the presence or absence of CCl_4. Metyrapone of 15 mmol/litter caused 50% reduction in pentane production with CCl_4.

DISCUSSION

Lieberman and Hochstein[6] reported that the rate of ethylene formation is similar to malondialdehyde formation in rat liver microsomes, caused by cuprous ions. Cohen and Lieberman[1] examined ethane formation in mouse liver and brain homogenates, and suggested that ethane formation is a useful index of lipid peroxidation *in vitro*. From these articles it has been confirmed that the formation of hydrocarbon gases occurs in parallel to that of malondialdehyde, this being a measure of lipid peroxidation.

Linolenic acid is a relatively selective precursor for ethane, while linoleic acid is a precursor for pentane[3,5]. Because of the quantitative dominance of the linoleic family in most animal tissue, Tappel and co-workers[2,9] recommended the measurement of pentane for this purpose.

From the results shown in Fig. 2—4, the optimal conditions for this experimental system were determined. Pentane formation is greatest under anaerobic conditions. 13.4 mmol/litter of CCl_4 and 2.1 mmol/litter of NADPH was used. Small amounts of pentane were detected without CCl_4. This formation of pentane may have been caused by NADPH-

dependent microsomal lipid peroxidation[4]. It has been reported that Vitamin E can reduce pentane and ethane formation induced by CCl_4 *in vivo*[2,5], and can reduce malondialdehyde formation in rat liver microsomes *in vitro*[8]. In this study vitamin E and GSH were added to the incubation system, and pentane formation was inhibited by both. SKF-525A, which is an inhibitor of P-450, inhibits malondialdehyde formation in rat liver microsomes *in vitro*[8]. Metyrapone, known as an inhibitor of P-450, also inhibited pentane formation. This is because the lipid peroxidation effect of CCl_4 is caused by the trichlorometyl radical produced by the CCl_4-Cl bond cleavage reaction of P-450[7].

The advantage of measuring hydrocarbon gases is that they are volatile at ambient temperatures, and stable and chemically inert[3]. Also, this method is simple and easy. With this system one can survey many substances which may activate lipid peroxidation via microsomal NADPH-P-450 enzyme systems or those which may inhibit it. The materials which may cause lipid peroxidation associated with microsomal enzyme systems must be added in place of CCl_4. Furthermore, the materials which may inhibit it must be added instead of GSH, Vitamin E and Metyrapone.

We have determined that the optimal conditions for pentane production via microsomal NADPH-P-450 enzyme systems require an anaerobic atmosphere, 2.1 mmol/litter of NADPH and 13.4 mmol/litter of CCl_4. Our results clearly show that pentane is formed by the free radical cleavage products of CCl_4 and that the measurement of pentane production can be used as an index of lipid peroxidation caused by microsomal enzyme systems.

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