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Stimulatory effect of anesthetics on dechlorination of carbon tetrachloride in guinea-pig liver microsomes

Kohyu Fujii*

Department of Anesthesiology and Critical Care Medicine, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima, Japan

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Abstract

Effects of the anesthetics isoflurane, enflurane, halothane and sevoflurane on the dechlorination of carbon tetrachloride to produce chloroform were investigated using guinea pig liver microsomes. Under anaerobic conditions, chloroform is produced from carbon tetrachloride by the microsomes in the presence of NADPH, and chloroform production from 86 μM carbon tetrachloride was enhanced to 146%, 133%, 123% and 115% by the addition of isoflurane, enflurane, halothane and sevoflurane, respectively. The half-life of oxidized cytochrome P450 which remained during the reduction by the addition of NADPH was shortened to 51%, 54%, 60% and 80% by isoflurane, enflurane, halothane and sevoflurane, respectively, without alteration of NADPH-cytochrome *c* reductase activity. These anesthetics hastened the onset of the 445 nm absorption band formation which was shown by microsomes with carbon tetrachloride in the presence of NADPH under anaerobic conditions. These results indicate that the anesthetics isoflurane, enflurane, sevoflurane and halothane stimulate the reduction of cytochrome P450 results in the acceleration of the carbon tetrachloride dechlorination. These results may have implications for other type II drugs that are administered during anesthesia.

Keywords: Anesthetics; Carbon tetrachloride; Chloroform; Dechlorination

1. Introduction

Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethylether), halothane (2-bromo-2-chloro-1,1,1-trifluoroethane), enflurane (2-chloro-1,1,2-

* Corresponding author, Tel: +082 257 5267; fax: +082 257 5269.

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trifluoroethyl difluoromethylether), sevoflurane (fluoromethyl-2,2,2-trifluoro-1-[trifluoromethyl]-ethylether) are halogenated anesthetics widely used for general anesthesia. Some halogenated compounds (halothane, methoxyflurane, chloroform, diethyl ether, isoflurane and chloroform) interact with the cytochrome P450 system and inhibit the aminopyrine *N*-demethylation (Van Dyke and Rikans, 1970; Korten and Van Dyke, 1973; Rahman et al., 1996). On the other hand, these halogenated compounds stimulate aniline hydroxylation (Wood and Wood, 1984; Van Dyke and Rikans, 1970; Korten and Van Dyke, 1973; Rahman et al., 1996). Among them, isoflurane accelerates not only aniline hydroxylation but also some halide metabolism, aerobic defluorination of 2-chloro-1,1-difluoroethane (Wang and Baker, 1993), and anaerobic dehalogenation of halothane (Rahman et al., 1994). This stimulatory effect of isoflurane was also observed in in vivo inhalation of isoflurane with halothane (Fiserova-Bergerova, 1974). Among these altered metabolisms, the dehalogenation of halothane and chloroform occurred under anaerobic conditions.

Isoflurane interacts with cytochrome P450 to stimulate the speed of reduction of cytochrome P450 without alterating cytochrome P450 reductase activity and results in the enhanced aniline hydroxylation. The stimulation of cytochrome P450 reduction may be linked to the enhancement of drug metabolism by isoflurane (Rahman et al., 1996). Because of the existence of few reports on the relationship between the stimulation of the cytochrome P450 reduction and the acceleration of metabolism by anesthetics, these relationships have remained unclear.

This study attempts to clarify the relationship between the stimulation of the speed of cytochrome P450 reduction and the enhancement of chloroform production from carbon tetrachloride by microsomal enzyme systems in the presence of anesthetics. We showed that the anesthetics isoflurane, enflurane, halothane and sevoflurane stimulate cytochrome P450 reduction which results in the acceleration of carbon tetrachloride dechlorination in the guinea pig liver microsomal mixed function oxidase system.

2. Materials and methods

This study was carried out according to the Guidance on Animal Experimentation in Research Facilities for Laboratory Animal Science, School of Medicine, Hiroshima University.

2.1. Reagents

Analytical grade carbon tetrachloride and chloroform were obtained from Katayama Chemical (Osaka, Japan). Isoflurane, enflurane, halothane and sevoflurane prepared for clinical use were obtained from Dynabot (Osaka, Japan), Dynabot (Osaka, Japan), Hoechst Japan (Tokyo, Japan), Maruishi (Osaka, Japan), respectively. All other reagents were of analytical grade.

2.2. Animals

Male Hartley guinea pigs weighing of 225–275 g were purchased from Kyushu Animals Center Co. (Oita, Japan) and housed in metal cages. The animals were starved for 24 h, sacrificed, and their livers excised. After perfusion through the portal vein with ice-cold physiological saline, the livers were homogenized in 0.05 M potassium phosphate buffer. Following centrifugation of the liver homogenate at $9000 \times g$ for 20 min, the supernatant was centrifuged again at $105\,000 \times g$ for 60 min. The resulting sediment was then suspended in 0.1 M potassium phosphate buffer which had been stored under nitrogen atmosphere after deoxygenation by degassing in a vacuum, and used as the liver microsomal suspension. All procedures after the preparation of microsomes were done under nitrogen atmosphere.

2.3. Assay of dehalogenation of carbon tetrachloride

After charging 12.3 ml butyl rubber-capped test tubes with nitrogen, the microsomes suspension (0.9–1.2 nmol of P450), nicotinamide adenine dinucleotide phosphate (NADPH; final concentration: 2.1 mM), carbon tetrachloride (final concentration: 86 μM), and 2 μmol anesthetics (isoflurane, enflurane, halothane or sevoflurane)

were added to a final volume of 1 ml. After 7 min incubation at 37°C, 0.5 ml of the gas phase was taken to measure chloroform by gas chromatography. Authentic chloroform was used as a standard.

2.4. Assay of cytochrome c reductase

The reaction system consisted of guinea pig liver microsomes (0.015–0.02 g wet liver), 0.6 mM of KCN and 0.33 mM of cytochrome *c* in 0.1 M potassium phosphate buffer, pH 7.4 with and without 10 μmol of the anesthetics (isoflurane, enflurane, halothane or sevoflurane) in a 4.9 ml cuvette. The reaction was started by the addition of a 30 μl aliquot of 0.01 M NADPH. The changes in absorbance at 550 nm were recorded by a spectrophotometer (Shimadzu UV-300, Kyoto, Japan). The experiment was performed at 25°C.

2.5. Assay of the cytochrome P450 reduction rate

Oxygen-free carbon monoxide was blown for 5 min into a 4.9 ml anaerobic cuvette, sealed with a rubber cap, containing 3.0 ml of liver microsomal suspension (0.3 g wet liver) in 0.1 M potassium phosphate buffer, pH 7.4. Two mmol isoflurane, enflurane, halothane or sevoflurane was injected into sample cuvette, except the control cuvette. A 50-μl aliquot of 0.05 M NADPH was injected through the rubber cap to initiate the reaction. To measure the CO-cytochrome P450 complex, changes in absorbance at 450 nm were recorded by Shimadzu UV-300 spectrophotometer at various times (P450v) until the reaction was complete. After removal of the rubber cap, a few crystals of sodium dithionite were added to both the experimental and reference cuvettes, and the absorbance were measured at both 450 nm and 490 nm to estimate total cytochrome P450 content (P450t). The difference between the total cytochrome P450 content (P450t) and the amount of CO-cytochrome P450 complex formation at various times (P450v) was the unreduced amount of cytochrome P450. The unreduced amounts of cytochrome P450 (P450t–P450v) were plotted on semilogarithmic paper. The rate constant of the initial phase, k, was estimated by logarithmic plots from the initial phase. The half-time, $T_{1/2}$, for the initial phase was calculated from the formula $t_{1/2} = 0.693/k$ (Sasame and Gillette, 1969). The experiment was performed at 25°C.

2.6. Assay of the 445 nm absorption band formation rate

In a 4.9 ml anaerobic cuvette sealed with a rubber cap containing 3.0 ml of liver microsomal suspension (0.3 g wet liver) in 0.1 M potassium phosphate buffer, pH 7.4, oxygen-free nitrogen was blown for 5 min. Isoflurane, enflurane, halothane or sevoflurane (2 μmol) and/or carbon tetrachloride (final concentration; 280 μM) were injected into the experimental cuvette through the rubber cap. A 50-μl aliquot of 0.05 M NADPH was injected into both experimental and reference cuvettes through the rubber caps. The changes in absorbance at 445 nm were recorded by a Shimadzu UV-300 spectrophotometer until the reaction was complete. The experiment was performed at 25°C.

2.7. Assay of protein microsomal

Protein contained in microsomes was measured using the method of Lowry et al. (1951).

2.8. Statistical analysis

The Bonferroni/Dunn test was used for statistical analysis of the results, with a P-value of less than 0.05 being considered significant.

3. Results

3.1. Effects of volatile anesthetics on the dechlorination of carbon tetrachloride

Under anaerobic conditions, carbon tetrachloride undergoes dechlorination in liver microsomes to produce chloroform in the presence of NADPH. The rate of chloroform formation by the reaction of 86 μM carbon tetrachloride and guinea pig liver microsomes in the presence of

NADPH was 0.0543 ± 0.0014 (mean ± S.D., n = 5) nmol/nmol P450/min. The formation rate was significantly accelerated to 123%, 146%, 115% and 133% of the control value by the addition of 2 μmol of each anesthetic (halothane, isoflurane, sevoflurane and enflurane), respectively (Fig. 1).

3.2. Effects of anesthetics on the NADPH-cytochrome P450 reductase

In the dechlorination of carbon tetrachloride, the reduction of heme of cytochrome P450 by NADPH-cytochrome P450 reductase is necessary. NADPH-cytochrome P450 reductase can reduce cytochrome c in the presence of NADPH. The cytochrome c reduction rate of guinea pig liver microsomes was 197 ± 4 nmol/min/mg (mean ± S.D., n = 5) protein. In the presence of halothane, isoflurane, sevoflurane and enflurane, the values were 198 ± 3, 197 ± 3, 197 ± 6 and 196 ± 3 nmol/min/mg protein (mean ± S.D., n = 5), respectively. Anesthetics were not affected by the cytochrome c reduction by guinea pig liver microsomes.

3.3. Effects of anesthetics on the cytochrome P450 reduction rate

The rate of cytochrome P450 reduction by

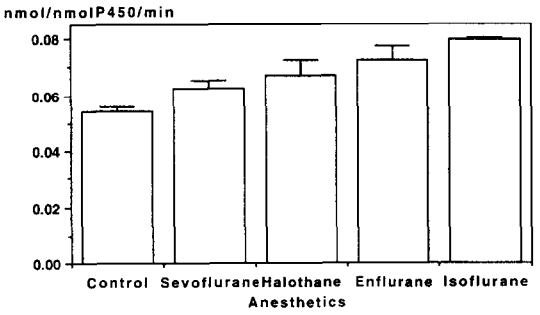

Fig. 1. Effects of anesthetics on the formation of chloroform. Reaction system: guinea pig liver microsomes (0.9–1.2 nmol of P450), NADPH (final concentration: 2.1 mM) carbon tetrachloride (final concentration: 86 μM), and anesthetic (2 μmol) in a final volume of 1 ml. After 7 min incubation at 37°C, 0.5 ml of the gas phase was taken to measure chloroform by gas chromatography. The production rate was significantly accelerated to 123%, 146%, 115% and 133% of the control value by the addition of 2 μmol of each anesthetics. Each value is the mean of five samples.

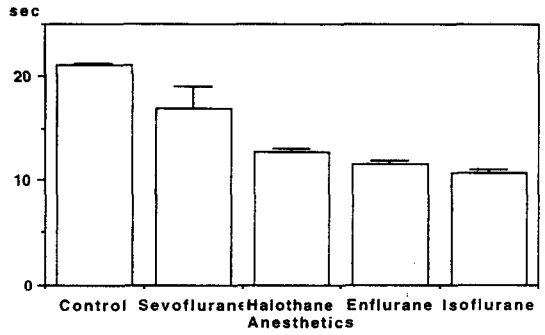

Fig. 2. Effect of anesthetics on the rate of cytochrome P450 reduction in guinea pig liver microsomes. The reduction rate of cytochrome P450 in guinea pig liver microsomal suspension (0.1 g wet liver) in a final volume of 3.0 ml of 0.1 M potassium phosphate buffer, pH 7.4, in the presence of anesthetic (2 μmol) was measured as described in the Materials and methods (Section 2). The rate constant, k, of the initial phase was determined from logarithmic plots. The half-time, $T_{1/2}$, for the initial phase was calculated from the formula $t_{1/2} = 0.693/k$. The data represent the mean and standard deviations of five samples. The half-life was shortened to 60%, 51%, 54% and 80% of the control value by 2 μmol of halothane, isoflurane, enflurane and sevoflurane, respectively. The experiment was performed at 25°C.

NADPH was estimated by the formation rate of carbon monoxide complex of cytochrome P450. The half-life of the oxidized form of cytochrome P450 remained unreduced by the addition of NADPH was 21.1 ± 0.11 s (mean ± S.D., n = 5) in the guinea pig liver microsome at 25°C. The half-life was shorted to 60%, 51%, 54% and 80% of the control value by the addition of 2 μmol of halothane, isoflurane, enflurane and sevoflurane, respectively (Fig. 2). Thus, volatile anesthetics tested in this study accelerated the rate of cytochrome P450 reduction.

3.4. Effects of anesthetics on the onset of the 445 nm absorption band

During the formation of chloroform from carbon tetrachloride, an intermediate complex which can be recognized by the spectrophotometer at 445 nm absorption is formed. The time of onset of the 445 nm absorption band was shortened to half by the addition of anesthetics (Fig. 3). The order of the stimulatory effect on the onset of the

445 nm absorption band formation is isoflurane, halothane, enflurane and sevoflurane.

4. Discussion

The stimulatory effect of isoflurane on the dechlorination of carbon tetrachloride in a guinea pig liver microsomes has been reported. The Michaelis constant (K_m) for the dechlorination of carbon tetrachloride was decreased, but the maximum velocity was unchanged in the presence of isoflurane (Fujii et al., 1996). So, the concentration of carbon tetrachloride used as a substrate of cytochrome P450 in this study to observe the difference of stimulation effect of anesthetics was 86 μM, lower value than the K_m value of 640 μM.

Drugs used as substrates for cytochrome P450 are classified as type I, type II and modified type II. Volatile anesthetics used in this study, halogenated ethers and a halogenated ethane, are classified type I substrates. Carbon tetrachloride is also classified as a type I substrate and catalyzed by the liver mixed function oxidase system, including cytochrome P450, to produce chloroform via a trichloromethyl radical which is produced by cleavage of the CCl_3-Cl bond. This radical also combines with the heme part of cytochrome P450 (Recknagel et al., 1980). The formation of chloroform was enhanced by the presence of volatile anesthetics (Fig. 1). A possible mechanism of the dechlorination of carbon tetrachloride is as follows: oxidized cytochrome P450 reacts with carbon tetrachloride to form a cytochrome P450-substrate complex. This type I complex is then reduced by NADPH-cytochrome P450 reductase. Carbon tetrachloride undergoes cleavage of the CCl_3-Cl bond resulting in formation of a trichloromethyl radical. The trichloromethyl radical binds to the heme part of cytochrome P450. This complex, which shows a modified type II spectrum, in turn subsequently decomposes with the formation of chloroform and oxidized cytochrome P450 (Recknagel et al., 1980). Thus, in anaerobic conditions, carbon tetrachloride may act like a type II substrate. Therefore, the dechlorination of carbon tetrachloride to produce chloroform may be stimulated by the type I substrate, volatile anesthetics. In fact, the anaerobic dehalogenation of halothane, a type I substrate which shows a modified type II spectrum during anaerobic dehalogenation, is also stimulated by isoflurane (Fujii, 1995). Further, the dechlorination of carbon tetrachloride is stimulated by acetone (Plaa et al., 1982), ethanol (Traiger and Plaa, 1971) and isopropyl alcohol (Anders and Harris, 1981; Plaa et al., 1982).

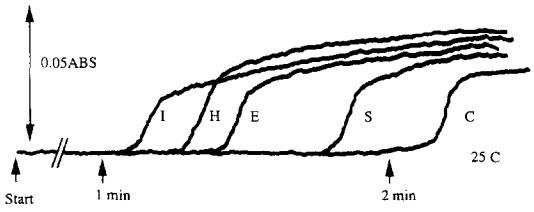

Fig. 3. Effects of anesthetics on the 445 nm absorption band formation rate. In an anaerobic cuvette (5 ml volume) sealed with a rubber cap containing 3.0 ml of liver microsomal suspension (0.3 g wet liver) in 0.1 M potassium phosphate buffer, pH 7.4, oxygen-free carbon monoxide was blown for 5 min, and anesthetics (2 μmol) and/or carbon tetrachloride (final concentration: 280 μM) were injected to the experimental cuvette through the rubber cap. A 50-μl aliquot of 0.05 M NADPH was injected into both the experimental and reference cuvette through the rubber cap. The changes in absorbance at 445 nm were recorded by Shimadzu UV-300 spectrophotometer until the reaction was completed.The experiment was performed at 25°C. C, control; I, isoflurane; H, halothane; E, enflurane; S, sevoflurane.

The anesthetics used in this study are halogenated ethers and a halogenated ethane which act as type I substrates for the liver microsomal cytochrome p450 system. Cytochrome P450 changes the heme state from low spin form to high spin form by combining with a type I substrate and is readily reduced by NADPH (Gigon et al., 1969). On the other hand, Sasame and Gillette (1969) reported that the type I substances did not alter the rate of cytochrome P450 reduction of *p*-nitrobenzoate reduction in mice liver microsomes. Thus, type I substances do not react uniformly to the reduction of cytochrome P450. In our study, the rate of cytochrome P450 reduction by NADPH in the guinea-pig liver microsomes was increased by volatile anesthetics which are type I substrates. The anesthetics in order of the stimulation of the cytochrome P450 reduction

were isoflurane, enflurane, halothane and sevoflurane (Fig. 2). This order did not correlate to the oil/gas partition ratios which for halothane, enflurane, isoflurane and sevoflurane were 185, 98.5, 94.0 and 53.9, respectively.

These potent volatile anesthetics also enhanced the anaerobic dehalogenation of carbon tetrachloride to produce chloroform (Fig. 1). Our results clearly supported the Castro's suggestion that the CCl4 activation is associated with the reduction of the CCl_4-cytochrome P450 complex mediated by cytochrome P450 reductase (Castro and Diaz-Gomez, 1972). Therefore, the degree of stimulation between the reduction of cytochrome P450 and the formation of chloroform might be similar, but, the relationship between the reduction of P450 and the dehalogenation of carbon tetrachloride did not completely correspond among the anesthetics. For example, halothane more readily reduced than sevoflurane (0.75 times), but stimulation of the formation of chloroform by halothane was only 0.07 times higher than by sevoflurane. Under anaerobic condition, the intermediate of halothane compete with the intermediate of carbon tetrachloride to combine with heme so that stimulation of dehalogenation of carbon tetrachloride might be negligible. In spite of the higher stimulation of carbon tetrachloride metabolism by isoflurane than enflurane, the effect of isoflurane on the cytochrome P450 reduction was negligible. This is the reason isoflurane has less effect on metabolism than enflurane (Chase et al., 1971; Hitt et al., 1974). The electron flow for the carbon tetrachloride metabolism may tend to degrade to enflurane metabolism more than isoflurane metabolism so the effect of isoflurane on the metabolism of carbon tetrachloride was larger than that of enflurane.

In the presence of anesthetics, the trichloromethyl radical-cytochrome P450 complex developed in less time after the addition of NADPH than in the absence of anesthetics. The alteration in the absorbance spectra at 445 nm after addition of NADPH reflects a change in either the structure or the kinetic properties of cytochrome P450 (Uehleke et al., 1973; Uehleke and Werner, 1975). Ascribing these effects to alterations in the activity of NADPH-cytochrome P450 reductase is not plausible because all anesthetics do not alter cytochrome *c* reductase. In order of the alteration, the anesthetics were correlated with the dechlorination of carbon tetrachloride without halothane. Because halothane also interacts with the heme of cytochlome P450 and shows an absorption band at 470 nm which affects the formation of the 445 nm, the 445 nm formation in the case of halothane, may not indicate the formation of trichloromethyl radical-cytochrome P450 complex. These results suggest that the facilitated-445 nm development is due to the acceleration of cytochrome P450 reduction by anesthetics. However, because the onset of the formation, not the rate altered, further study is required.

Our results suggest that the stimulation of the cytochrome P450 reduction by anesthetics is linked to the stimulatory effect of anesthetics on the metabolism of type II substrate. Moreover, the effect depends not only on the reduction of cytochrome P450 but also its, the anesthetic's, metabolic rate.

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References

Anders, M.W. and Harris, R.N. (1981) Effect of 2-propanol treatment on carbon tetrachloride metabolism and toxicity. Adv. Exp. Med. Biol. 136, 591–602.

Castro, J.A. and Diaz-Gomez, M.I. (1972) Studies on the irreversible binding of ^{14}C-CCl_4 to microsomal lipids in rats under varying experimental conditions. Toxicol. Appl. Pharmacol. 23, 541–552.

Chase, R.A., Holaday, D.A., Fiserova-Bergerova, V., Saidman, L.J. and Mack, F.E. (1971) The biotransformation of ethrane in man. Anesthesiology 35, 262–267.

Fiserova-Bergerova, V. (1974) Inhibitory effect of isoflurane upon oxidative metabolism of halothane. Anaesth. Analg. 63, 399–404.

Fujii, K. (1995) Isoflurane acts as an inhibitor on oxidative dehalogenation while acting as an accelerator on reductive dehalogenation of halothane in guinea pig liver microsomes. Toxicology 104, 123–128.

Fujii, K., Rahman, M.M. and Yuge, O. (1996) Isoflurane enhances dechlorination of carbon tetrachloride in guinea pig liver microsomes. J. Appl. Toxicol. 16, 249–253.

Gigon, P.L., Gram, T.E. and Gillette, J.R. (1969). Studies on the rate of reduction of hepatic microsomal cytochrome P450 by reduced nicotinamide adenine dinucleotide phosphate: effect of drug substrates. Mol. Pharmacol. 5, 109–122.

Hitt, B.A., Mazee, R.I., Cousins, M.J., Edmunds, H.N., Barr, G.A. and Trudell, J.R. (1974). Metabolism of isoflurane in Fischer 344 rats and man. Anesthesiology 40, 62–67.

Korten, R.A. and Van Dyke, R.A. (1973). Acute interaction of drugs. 1. The effect of volatile anesthetics on the kinetics of aniline hydroxylase and aminopyrine demethylase in rat hepatic microsomes. Biochem. Pharmacol. 22, 2105–2112.

Lowry, O.H., Rosebourg, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Plaa, G.L., Hewitt, W.R., du Souich, P., Caille, G. and Lock, S. (1982) Isopropanol and acetone potentiation of carbon tetrachloride-induced hepatotoxicity: single versus repetitive pretreatments in rats. J. Toxicol. Environ. Health 9, 235–250.

Rahman, M.M., Fujii, K., Sato, N. and Yuge, O. (1994) Isoflurane increases the anaerobic metabolism of halothane. J. Appl. Toxicol. 14, 43–46.

Rahman, M.M., Fujii, K., Kawamoto, M. and Yuge, O. (1996) Contrasting effect of isoflurane on drug metabolism: decreased type I and increased type II substrate metabolism in guinea pig liver microsomes. J. Appl. Toxicol. 15 (in press).

Recknagel, R.O., Glende Jr, E.A. and Hruszkewycz, A.M. (1980) Chemical mechanisms in carbon tetrachloride toxicity. In: W.A. Pryor (Ed), Free Radicals in Biology. Academic Press, New York, Vol. 3, pp. 97–132.

Sasame, H.A. and Gillette, J.R. (1969) Studies on the relation between the effects of various substances on absorption spectrum of cytochrome P450 and the reduction of *p*-nitrobenzoate by mouse liver microsomes. Mol. Pharmacol. 5, 123–130.

Traiger, G.J. and Plaa, G.L. (1971) Differences in the potentiation of carbon tetrachloride in rats by ethanol and isopropanol pretreatment. Toxicol. Appl. Pharmacol. 20, 105–112.

Van Dyke, R.A. and Rikans, L.E. (1970) Effect of the volatile anesthetics on aniline hydroxylase and aminopyrine demethylase. Biochem. Pharmacol. 19, 1501–1502.

Wood, M. and Wood, A.J.J. (1984) Contrasting effect of halothane, isoflurane, a d enflurane on in vitro drug metabolism in the rat. Anesth. Analg. 63, 709–714.

Wang, Y. and Baker, M.T. (1993) NADPH and oxygen consumption in isoflurane-facilitated 2-chloro-1,1-difluoroethane metabolism in rabbit liver microsomes. Drug Metab. Dispos. 21, 299–304.

Uehleke, H. and Werner, T. (1975) A comparative study on the irreversible binding of labeled halothane trichlorofluoromethane, chloroform and carbon tetrachloride to hepatic protein and lipids in vitro and in vivo. Arch. Toxicol. 34, 289–3089.

Uehleke, H., Hellmer, K.H. and Tabarelli-Poplawski, S. (1973) Metabolic activation of halothane and its covalent binding to liver endoplasmic proteins in vitro. Naunyen-Schmiedeberg's Arch. Pharmacol. 279, 39–52.