Hiroshima J. Med. Sci.
Vol.40, No.1, 23~28, March, 1991
HIJM 40-4

Aerobic Dehalogenation of Halothane Showing Different Substrate Dependency from Anaerobic Dehalogenation in Liver Microsomes of Guinea Pig.

Miwako NAKAO, Kohyu FUJII, Hiroyuki KINOSHITA,
Osafumi YUGE and Michio MORIO

Department of Anesthesiology and Critical Care Medicine, Hiroshima University School of Medicine
1-2-3, Kasumi, Minami-ku, Hiroshima 734, Japan

ABSTRACT

The formation of trifluoroacetic acid (TFAA) from halothane under aerobic conditions and that of chlorotrifluoroethane (CTE) and chlorodifluoroethylene (CDE) from halothane under anaerobic conditions were studied using guinea pig liver microsomes. The formation of TFAA was inhibited by specific inhibitors of cytochrome P450 (P450), such as carbon monoxide and metyrapone and was dependent upon P450 contents. The maximum activity of the TFAA formation was obtained at pH 6.0. On the other hand, the maximum activity to form CTE and CDE was obtained at pH 7.4. The formation of TFAA reached a plateau at a halothane concentration above 0.17 mM, but the rate of formation of CDE and CTE was dependent upon a halothane concentration up to 1.5 mM. The values of apparent Michaelis-Menten constant (Km) and maximum velocity (Vmax) for TFAA formation were 0.067 mM and 0.349 nmol/nmol P450/min respectively, those for CDE formation were 0.983 mM and 0.326 nmol/nmol P450/min respectively, and those for CTE formation were 1.71 mM and 0.752 nmol/nmol P450/min respectively. These results showed clearly that the formation of TFAA, CDE and CTE was catalyzed by the P450 system in guinea pig liver microsomes. Under optimal conditions, saturation was observed in the formation of TFAA from halothane at a halothane concentration above 0.17 mM but the formation of CDE and CTE was not saturated at this concentration, and the value of apparent Km for TFAA formation was lower than those for CDE and CTE formation.

Key words: *Anesthetics; halothane*
Biotransformation; trifluoroacetic acid,
Microsomes; liver, guinea pig

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is a potent volatile anesthetic widely used in clinical practice. Halothane anesthesia is occasionally followed by liver disorder (halothane hepatitis)[15]. While the mechanism still remains uncertain, many reports have recently been published that halothane metabolites are related to halothane hepatitis [7,13]. It is therefore important to clarify the metabolic pathway of halothane.

Halothane undergoes aerobic dehalogenation to trifluoroacetic acid (TFAA)[4] and anaerobic dehalogenation to chlorotrifluoroethane (CTE) and chlorodifluoroethylene (CDE) by the microsomal cytochrome P450 (P 450) system [1]. Sawyer first reported that the metabolic rate of halothane in the liver was not dose dependent on alveolar halothane concentration [14]. Indeed, Okida reported that biliary TFAA concentration was highest at a subanesthetic concentration of halothane in rabbit *in vivo* and there was no notable increase at higher concentrations [11]. However, the excreted anaerobic metabolites of halothane in expired gas, such as CTE and CDE, increased in a concentration-dependent manner over anesthetic halothane concentration range. Therefore it is necessary to determine whether the TFAA excretion system or the TFAA formation system limits the total amount of biliary TFAA at a subanesthetic halothane concentration. In order to elucidate the possible mechanisms of biliary TFAA regulation at a subanesthetic concentration of halothane, *in vitro* study was conducted using guinea pig liver microsomes.

MATERIALS AND METHODS

1) Materials

TFAA was obtained from Katayama Chemical (Japan). Halothane was purchased from Hoechist (Japan) and NADPH from Oriental Yeast Co. (Japan). All the other reagents were of the highest grade commercially available.

2) Animals

Adult male guinea pigs, weighing about 250 g each, were used. After 24-hour fasting, the guinea pigs were sacrificed by decapitation and the liver

was immediately excised. After irrigation with 4°C physiologic saline through the portal vein to wash out the blood, the liver was homogenized in 0.1 M potassium phosphate buffer solution at pH 7.4. The subcellular fraction was performed according to the modified Hogeboom's method [2].

3) Assay of P450

The quantity of P450 was measured by spectrophotometer (Shimazu UV 300, JAPAN) following the procedure of Omura and Sato [11].

4) Incubation systems

Under aerobic conditions, the incubation system consisted of microsomal suspension (total volume of 1 ml, 5 nmol/ml P450 and pH 6.0, 0.1 M potassium phosphate buffer), NADPH (10 μmol) and halothane (10 μmol). Under anaerobic conditions the incubation system consisted of microsomal suspension (total volume of 1 ml, 1 nmol/ml P450, 0.1 M potassium phosphate buffer at pH 7.4), NADPH (10 μmol) and halothane (10 μmol). In some experiments oxygen was replaced with carbon monoxide (CO) or metyrapone was added, both of which are specific inhibitors of P450 in the incubation system. The optimal condition for TFAA, CDE and CTE measurement was sought by varying the individual parameters of the incubation system, such as pH, concentration of P450 and duration of halothane exposure.

5) Assay of dehalogenation of halothane.

Under aerobic conditions, oxygen was sealed in a 60 ml silicon-capped flask containing the microsomal suspension. Under anaerobic conditions, deoxygenated nitrogen was sealed in a 13.5 ml silicon-capped test tube. After preincubation in a water bath at 37°C for 10 min, halothane was added and then the reaction was initiated by adding NADPH. This mixture was incubated for 60 min under aerobic condition and for 10 min under anaerobic condition. The aerobic reaction was terminated by a deproteinization process, i.e. protein was removed from the mixture by ultrafiltration at 4°C for 30 min. Aerobic metabolite, TFAA, was analyzed with ion exchange chromatography (Yokogawa Electric Co. Model IC-100, JAPAN), which was equipped with an electric conductivity detector. The analytical condition of ion exchange chromatography for TFAA was determined by Kawaguchi's method [5]. Under anaerobic conditions, a part of the mixture's gas phase was subjected to flame ionization gas chromatography (Shimadzu Co. GC-4A, JAPAN) for analysis of CDE and CTE. The operating condition for gas chromatography was determined following the method of Mukai [7].

RESULTS

1) Effect of CO and metyrapone on the aerobic dehalogenation of halothane to TFAA (Table 1-a) and on the anaerobic dehalogenation of halothane to CDE and CTE (Table 1-b).

Table 1-a, 1-b. Effect of carbon monoxide (CO) and metyrapone on aerobic dehalogenation of halothane to TFAA and on anaerobic dehalogenation of halothane to CDE and CTE.

Under aerobic conditions, oxygen was replaced with CO, or metyrapone was added to the incubation system. The CO/O_2(v/v) ratio in the flask was between 1: 1 and 6:1. The incubation system consisted of microsomal suspension (total volume of 1 ml, 5 nmol/ml P450 and pH 6.0, 0.1 M potassium phosphate buffer), NADPH (10 μmol) and halothane (10 μmol).

Under anaerobic conditions, CO or metyrapone was added to the incubation system, which consisted of microsomal suspension (total volume of 1 ml, 1 nmol/ml P450 and pH 7.4, 0.1 M potassium phosphate buffer), NADPH (10 μmol) and halothane (10 μmol).

CO/O_2 (%)	Inhibition of TFAA formation (%)
0	0
50	47
66.7	61
85.7	68
metyrapone (mM)	
0	0
10	42
75	83
100	88

CO/N_2 (%)	Inhibition of CDE	CTE (%)
0	0	0
0.15	68.9	70.2
0.3	77.5	77.6
0.6	86.7	91.6
1.0	100	97.9
metyrapone (mM)		
3.25	91.1	100

Replacement of CO (50-86%) in the incubation system under aerobic conditions inhibited the formation of TFAA from halothane in the liver microsomes. The degree of inhibition was almost proportional to the CO/O_2 ratio. Addition of metyrapone to the aerobic incubation system inhibited the rate of TFAA formation from halothane in a similar manner. Addition of CO (0.15-1.0%) to the incubation system under anaerobic conditions inhibited the formation of CDE and CTE from halothane. Addition of metyrapone to the anaerobic incubation system also inhibited the rate of CDE and CTE formation from halothane.

2) Time course of the formation of TFAA, CDE and CTE from halothane in liver microsomes (Figs. 1 and 2).

As shown in Fig. 1, the rate of TFAA formation from halothane increased linearly up to 2 hours. Fig. 2 shows the rate of CDE and CTE formation from halothane in liver microsomes. The rate increased linearly up to 30 min.

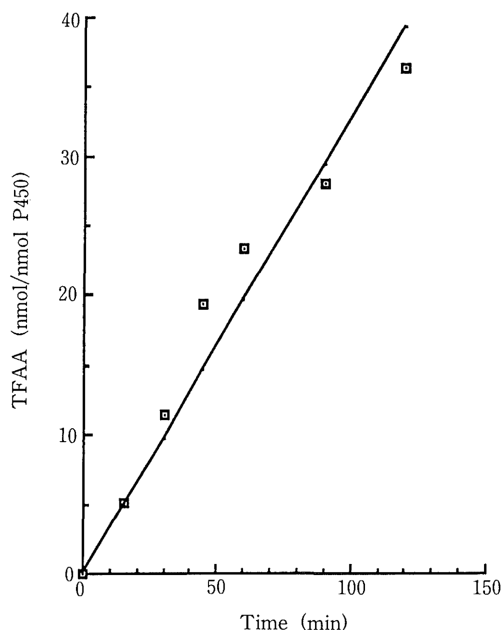

Fig. 1. Time course of the formation of TFAA from halothane in liver microsomes. The incubation system consisted of microsomal suspension (total volume of 1 ml, 5 nmol/ml P450 and pH 6.0, 0.1 M potassium phosphate buffer), NADPH (10 μmol) and halothane (10 μmol). Reaction time was up to 2 hours. A positive correlation between time and formation of TFAA was observed. ($p<0.001$)

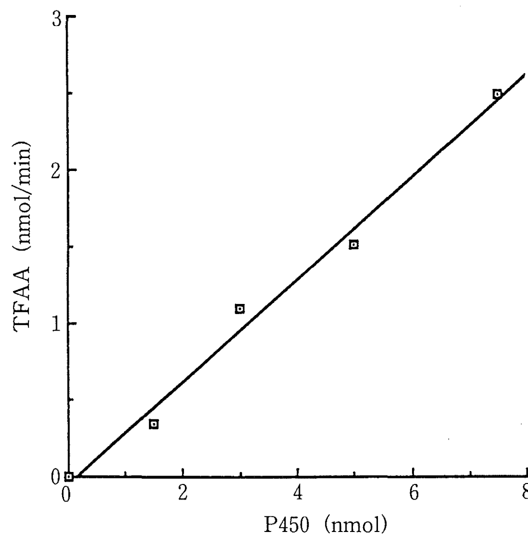

Fig. 3. Relationship between the rate of TFAA formation from halothane and P450 concentration in liver microsomes. Aerobic reactions were made by the incubation system containing 1.5 to 7.5 nmol P450. NADPH (10 μmol / 5 nmol P450) and halothane (10 μmol) were added to the microsomal suspension (pH 6.0, 0.1 M potassium phosphate buffer). Positive correlation between P450 concentration and the rate of TFAA formation was observed. ($p<0.001$)

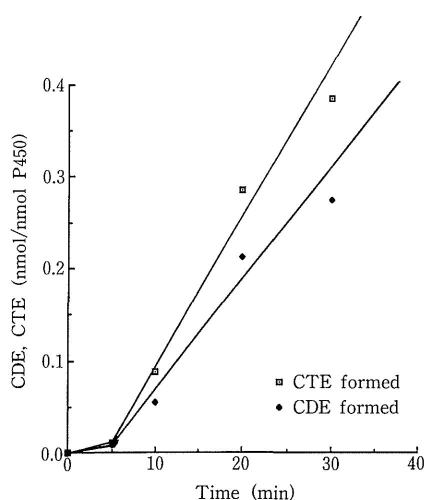

Fig. 2. Time course of the formation of CDE and CTE from halothane in liver microsomes. The incubation system consisted of microsomal suspension (total volume of 1 ml, 1 nmol/ml P450 and pH 7.4, 0.1 M potassium phosphate buffer), NADPH (10 μmol) and halothane (10 μmol). Reaction time was up to 30 min. Positive correlation between time and formation of CDE and CTE was observed. (CDE: $p<0.001$; CTE: $p<0.001$)

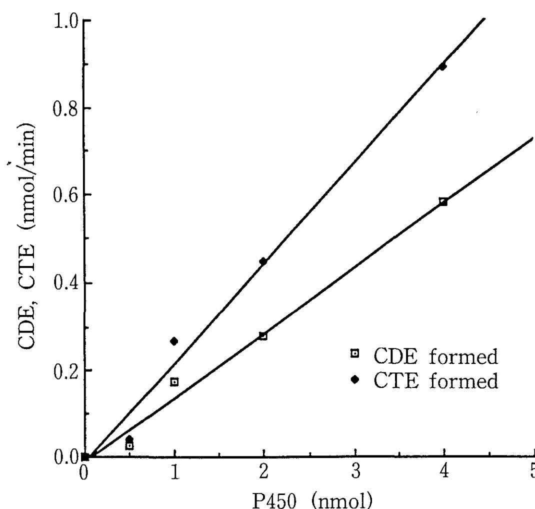

Fig. 4. Relationships between P450 concentration in liver microsomes and the rate of CDE and CTE formation from halothane. Anaerobic reaction was made by the incubation system containing 0.5-4 nmol P450. NADPH (10 μmol/nmol P450) and halothane (10 μmol) were added to the microsomal suspension (pH 7.4, 0.1 M potassium phosphate buffer). Positive correlation between P450 concentration and the rate of CDE and CTE formation was observed. (CDE: $p<0.001$; CTE: $p<0.001$)

3) The relationship between P450 concentration in liver microsomes and the rate of TFAA, CDE and CTE formation from halothane (Figs. 3 and 4).

As shown in Figs. 3 and 4, the rate of TFAA, CDE and CTE formation from halothane increased proportionally to the P450 concentration in liver microsomes.

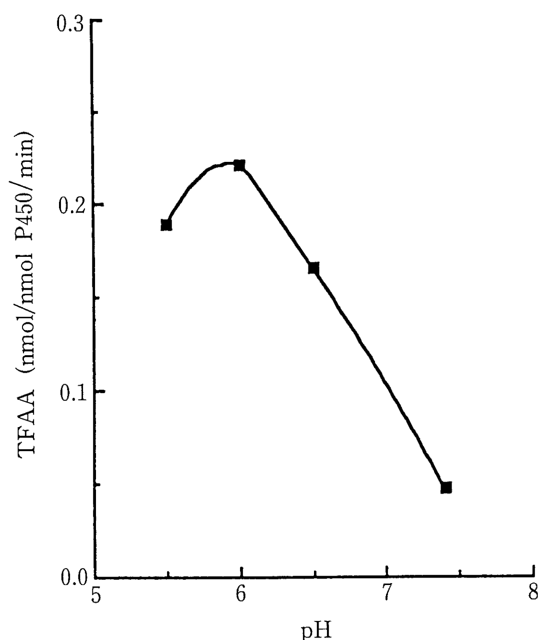

Fig. 5. Optimal pH value of aerobic dehalogenation of halothane. pH of 0.1 M potassium phosphate buffer was varied from 5.5 to 7.4. The incubation system consisted of microsomal suspension (total volume of 1 ml, 5 nmol/ml P450 and 0.1 M potassium phosphate buffer), NADPH (10 μmol) and halothane (10 μmol).

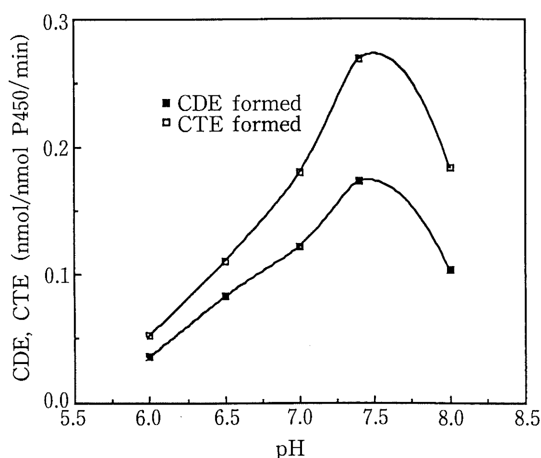

Fig. 6. Optimal pH value of anaerobic dehalogenation of halothane. The pH of 0.1 M potassium phosphate buffer was varied from 5.5 to 7.4. The incubation system consisted of microsomal suspension (total volume of 1 ml, 1 nmol/ml P450 and 0.1 M potassium phosphate buffer), NADPH (10 μmol) and halothane (10 μmol).

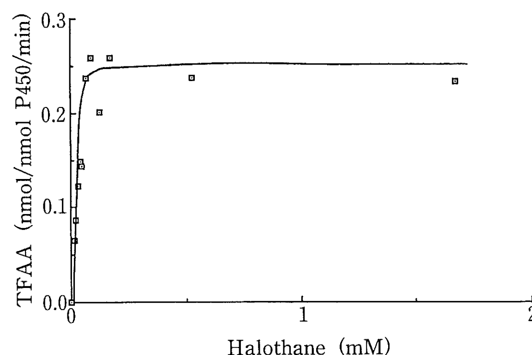

Fig. 7. Relationship between halothane concentration and the rate of TFAA formation from halothane. Under aerobic conditions, halothane concentration was varied from 0.017 to 1.67 mM. The incubation system consisted of microsomal suspension (total volume of 1 ml, 5 nmol/ml P450 and pH 6.0, 0.1 M potassium phosphate buffer), NADPH (10 μmol) and halothane. The values of apparent Km and Vmax for TFAA formation were 0.067 mM and 0.349 nmol/nmol P450/min.

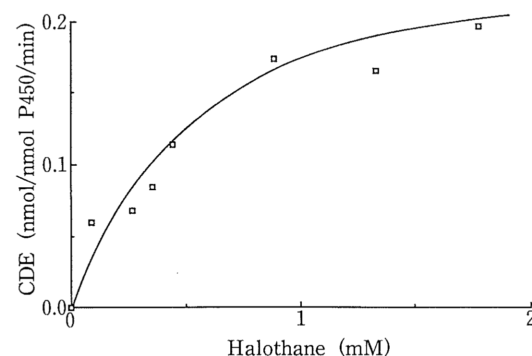

Fig. 8. Relationship between halothane concentration and the rate of CDE formation from halothane under anaerobic conditions. Instead of oxygen, deoxygenated nitrogen was sealed in the silicon-capped test tube. Under anaerobic conditions, halothane concentration was varied from 0.037 to 1.778 mM. The incubation system consisted of microsomal suspension (total volume of 1 ml, 1 nmol/ml P450, and pH 7.4, 0.1 M potassium phosphate buffer), NADPH (10 μmol) and halothane. The values of apparent Km and Vmax for CDE formation were 0.983 mM and 0.326 nmol/nmol P450/min.

4) Optimal pH value (Figs. 5 and 6).

As shown in Figs. 5 and 6, the optimal pH value was 6.0 for the aerobic dehalogenation of halothane to TFAA and 7.4 for the anaerobic dehalogenation of halothane. Thus, 0.1 M potassium phosphate buffer at pH 6.0 was used in the aerobic experiments, and 0.1M potassium phosphate buffer at pH 7.4 was used in the anaerobic reaction.

5) The relationship between halothane concentration and the rate of TFAA, CDE and CTE formation from halothane (Figs. 7, 8 and 9).

Figs. 7, 8 and 9 show the relationship between halothane concentration and the rate of TFAA, CDE and CTE formation from halothane, respectively. The rate of TFAA formation reached a plateau when the halothane concentration was above 0.17 mM, but the rates of formation of CDE and CTE were dependent upon a halothane concentration up to 1.5 mM.

The values of apparent Km and Vmax for TFAA formation were 0.067 mM and 0.349 nmol/nmol

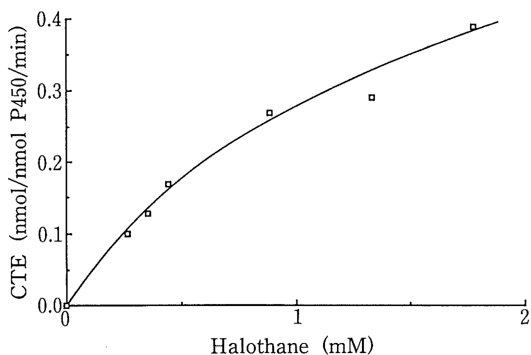

Fig. 9. Relationship between halothane concentration and the rate of CTE formation from halothane under anaerobic conditions. Experimental conditions were the same as in Fig. 8. The values of apparent Km and Vmax for CTE formation were 1.71 mM and 0.752 nmol/nmol P450/min.

P450/min, and those for CDE formation were 0.983 mM and 0.326 nmol/nmol P450/min. The values of apparent Km and Vmax for CTE formation were 1.71 mM and 0.752 nmol/nmol P450/min. These values were determined by the Lineweaver-Burk plot, respectively.

DISCUSSION

Halothane associated liver damage has been grouped into two distinct entities, the mild type and the severe type [10]. In the severe type of halothane hepatotoxicity, the hepatic reaction is usually delayed and the clinical course is often lethal. The rarity of the disease, its association with repeated administrations of halothane, and the difficulty in reproducing it in animals suggest an immune mediated mechanism [3]. Vergagni [7] strongly indicated that halothane hepatitis was an immune mediated drug toxicity. This hepatitis was characterized by the presence of serum antibodies specific to liver microsomal proteins. These antibodies were altered covalently by trifluoroacetyl halide, an oxidative intermediate metabolite from halothane to TFAA. TFAA, an aerobic metabolite of halothane, was excreted in urine for two weeks after halothane anesthesia, and the amount of excreted TFAA was greater on the postoperative day rather than during halothane anesthesia [8,16]. This means that trifluoroacetyl halide is exposed to the immune system for a long time.

The causes of this prolonged elimination of TFAA after halothane anesthesia are yet unknown. Kinoshita has reported that the enterohepatic circulation of TFAA is one candidate [6]. Another cause may be the continued dehalogenation of halothane after halothane anesthesia. Okida et al simultaneously measured both the biliary excretion of TFAA and the pulmonary excretion of CDE and CTE during 5 hours of inhalation at various concentrations of halothane (0.02-1.5%) [5] *in vivo*. They showed that the biliary TFAA level reached a plateau at 0.05% of halothane concentration which is less than 1/10 of the anesthetic halothane concentration, but no notable increase of the amount of TFAA in the bile was observed at higher halothane concentrations. This result suggests that a considerable amount of halothane is possibly metabolized to TFAA for a long period at such a trace concentration of halothane that halothane is redistributed into the blood from the fat tissue after halothane anesthesia. However, they were not able to clarify the reasons why there were no changes in the total amount of biliary TFAA at higher halothane concentrations. In *in vivo* experiments, a possibility exists that excretion of biliary TFAA is limited by the excretion system and that the biliary TFAA level cannot increase in concentration dependent manner above the subanesthetic halothane concentration.

Our study clearly showed that the aerobic and anaerobic dehalogenation of halothane were catalyzed by the P450 system of guinea pig liver microsomes (Table 1). Next the optimal conditions for *in vitro* aerobic and anaerobic dehalogenation of halothane were determined. Under optimal conditions it was found that the rate of formation of TFAA was already saturated above the subanesthetic concentration of halothane, but the formation of CDE and CTE was not saturated at this concentration (Figs. 7, 8 and 9), and the value of apparent Km for TFAA formation was lower than those for CDE and CTE formation. It is therefore concluded that saturation of aerobic dehalogenation of halothane was one of the reasons why there was no increase in the biliary TFAA level in concentration dependent manner above the subanesthetic halothane concentration. Continued TFAA formation from dissolved halothane in fat tissue after halothane anesthesia may be responsible for the prolonged elimination of TFAA into urine.

ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-aid for Science Research from the Ministry of Education, Science and Culture of Japan and the Association for the Advancement of Medicine of the Tsuchiya Foundation.

(Received August 28, 1989)
(Accepted December 19, 1990)

REFERENCES

1. **Fujii K., Miki M., Sugiyama T., Morio M., Yamano T. and Miyake Y.** 1981. Anaerobic dehalogenation of halothane by reconstituted liver microsomal cytochrome P450 enzyme system. Biochem. Biophys. Research Commun. **102**: 507–512.
2. **Hogeboom, G.H.** 1957. Fractionation of cell components of animal tissues. Methods in Enzymology **1**: 16–19.
3. **Hubbard, A.K., Gandolfi, A.J. and Brown, B.R.**

1988. Editorial. Immunological basis of anesthetic-induced hepatotoxicity. Anesthesiol. **69**: 814–817.
4. **Karashima, D., Hirokata, Y., Shigematu, A. and Furukawa, T.** 1977. The in vitro metabolism of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) by hepatic microsomal cytochrome P-450. J. Pharmacol. Exp. Ther. **203**: 409–416.
5. **Kawaguchi, R., Fujii, K. and Morio, M.** 1989. Determination by ion-exchange chromatography of isoflurane in the rabbit. Hiroshima J. Med. Sci. **38**: 27–34.
6. **Kinoshita, H.** 1989. Enterohepatic circulation of trifluoroacetic acid - A pharmacokinetic study-. Hiroshima J. Anesth. **25**: 155–164.
7. **Lind,R.C., Gandolfi, A.J., Hall, P. de la M. and Brown, Jr., B.R.** 1988. Role of oxidative biotransformation of halothane in the guinea pig model of halothane-associated hepatotoxicity. Anesthesiol. **69**: A438.
8. **Morio, M., Fujii, K., Takiyama, R., Chikasue, F., Kikuchi, H. and Ribari'c L.** 1980. Quantitative analysis of trifluoroacetate in the urine and blood by isotachophoresis. Anesthesiol. **53**: 56–59.
9. **Mukai, S., Morio, M., Fujii, K. and Hanaki, C.** 1977. Volatile metabolites of halothane in the rabbit. Anesthesiol. **47**: 248–251.
10. **Neurberger, J. and Williams, R.** 1984. Halothane anaesthesia and liver damage. Br. Med. J. **289**: 1136–1139.
11. **Okida, M., Kikuchi, H. and Fujii, K.** 1986. Concentration-dependence of halothane metabolism in rabbits. Hiroshima J. Med. Sci. **35**: 15–20.
12. **Omura, T. and Sato, R.** 1964. The carbon monoxide-binding pigment of liver microsomes. J. Biol. Chem. **239**: 2370–2378.
13. **Sato, N., Fujii, K., Yuge, O and Morio, M.** 1990. The Association of halothane-induced lipid peroxidation with the anaerobic metabolism of halothane: An *in vitro* study in guinea pig liver microsomes. Hiroshima J. Med. Sci. **39**: 1–6.
14. **Sawyer, D.C., Eger, E.I., Bahlman, S.H. and Cullen, B.F.** 1971. Concentration dependence of hepatic halothane metabolism. Anesthesiol. **34**: 230–235.
15. **Subcommittee on the National Halothane Study of the Committee on Anesthesia National Academy of Sciences-National Research Council.** 1966. Summary of the National Halothane Study. Possible association between halothane anesthesia and postoperative necrosis. JAMA. **197**: 775.
16. **Takiyama, R., Morio, M., Fujii, K., Kikuchi, H., Yuge, O., Chikasue, F., Taira, Y. and Jordanov, G.** 1985. Clinical effects of halothane concentration on trifluoroacetic acid excretion in urine. Hiroshima J. Med. Sci. **34**: 377–380.
17. **Vergani, D., Vergani, G.M., Alberti, A., Neuberger, J., Eddleston, A.L.W.F., Devis, M. and Williams, R.** 1980. Antibodies to the surface of halothane-altered rabbit hepatocytes in patients with severe halothane-associated hepatitis. New Eng. J. Med. **303**: 66–71.