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Contrasting Effect of Isoflurane on Drug Metabolism: Decreased Type I and Increased Type II Substrate Metabolism in Guinea Pig Liver Microsomes*

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Key words: anaesthetic; isoflurane; type I substrate; type II substrate; oxidative metabolism; aminopyrine N-demethylation; aniline hydroxylation; hepatic microsome; guinea pig.

Inhalation anaesthetics might affect perioperative drug elimination by altering drug distribution, hepatic blood flow or drug metabolism. The *in vitro* effects of isoflurane on aniline hydroxylation and aminopyrine N-demethylation were investigated with guinea pig liver microsomes to assess the role of isoflurane on oxidative drug metabolism through the hepatic mixed-function oxidase system.

***p*-Aminophenol and formaldehyde were measured spectrophotometrically as metabolic products of aniline hydroxylation and aminopyrine N-demethylation, respectively, where the reaction mixture consisted of a microsomal suspension, NADPH, aminopyrine or aniline, with or without isoflurane. The rate of cytochrome P–450 reduction by NADPH affected in the presence of isoflurane was investigated by spectrometric measurement of the CO–cytochrome P–450 complex formation at various times.**

Due to the addition of isoflurane, the V_{max} values for aniline hydroxylation evidently increased except in high isoflurane concentration (3.33 mM) and for aminopyrine N-demethylation the value was significantly low only in the presence of a high isoflurane concentration, whereas the K_m values significantly decreased in aniline hydroxylation and increased in aminopyrine N-demethylation, and isoflurane also accelerated the rate of cytochrome P–450 reduction by NADPH.

These results reflect the inhibition of aminopyrine N-demethylation and activation of aniline hydroxylation in the presence of isoflurane as a consequence of isoflurane-accelerated cytochrome P–450 reduction by NADPH and/or drug–enzyme binding properties, and may have implications on the metabolism of perioperatively administered drugs during isoflurane anaesthesia.

INTRODUCTION

It is common in medicine for a patient to take more than one medication and this is also applicable at the time of general anaesthesia. Inhalation anaesthetic agents may influence drug disposition by three mechanisms: first, by acute alteration in drug distribution when changes in drug binding or volume of distribution occur; second, by changes in hepatic blood flow; and third, by changes in drug-metabolizing ability of the liver. Since metabolism is one means that the biological system has of limiting the duration of action of a drug, it is interesting to determine the effect of the presence of volatile anaesthetics on the metabolism of other drugs.

Several volatile anaesthetics and other organic solvents have been found to undergo metabolism by an enzyme system very similar to, or the same as, the hepatic mixed-function oxidase system,[1] which carries out a large number of drug biotransformations.

According to the substrate–enzyme binding spectrum, drugs are mainly of two types: type I and type II. Depending on oxygen tension, biotransformation occurs through two different pathways: oxidative and, rarely, reductive. Investigators have found that inhalation anaesthetics, in common with a variety of non-volatile drugs, require microsomal enzymes, reduced NADPH and molecular oxygen.[2] Therefore, it can be predicted that an interaction between volatile anaesthetics and non-volatile drugs may occur, as they appear to be metabolized by the same cytochrome P–450 enzyme system.

Wood and Wood[3] reported from their *in vivo* findings in rats that halothane inhibited the rate of aminopyrine (type I substrate) elimination in a dose-dependent fashion, whereas isoflurane had a smaller, short-lasting effect and enflurane did not significantly affect the elimination rate. Van Dyke and Rikans suggested that halothane, methoxyflurane and the trichloroethane isomers stimulated aniline (type II substrate) hydroxylase activity, while there was no significant effect on aminopyrine demethylase.[4]

Chronic exposure to inhalation anaesthetics has been implicated to cause stimulation of drug-metabolizing activity.[5,6] The reactions stimulated by volatile anaesthetics (type I substrate) are limited to the oxidation of certain type II substrates: aniline and zoxazolamine.[7] However, we reported previously that the production of the anaerobic metabolites of halothane was increased

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by isoflurane,[8] although halothane and isoflurane are both type I substrates.

Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) is a widely used volatile inhalation anaesthetic. Physicochemically isoflurane is stable, which can be presumed from its resistance to biodegradation: only less than 0.2% is biodegraded to metabolites that are excreted in urine as fluoride and trifluoroacetic acid.[9] In addition, with this near-absence of isoflurane metabolism, data from animal and human studies suggest that isoflurane may not cause either nephrotoxicity or hepatotoxicity, because the importance of anaesthetic metabolism lies in the association between metabolism and liver and kidney toxicity. The low degree of isoflurane metabolism is to be without biological significance and it is not expected to interfere with the biotransformation of other xenobiotics. Fujii *et al.* also performed an *in vitro* study on carbon tetrachloride metabolism to chloroform that demonstrated enhancement by isoflurane,[10] where carbon tetrachloride metabolism appears to be similar to reductive halothane metabolism.

The present study, therefore, is our attempt to describe the effects of a less-metabolized volatile anaesthetic, isoflurane on the K_m (Michaelis constant: concentration of substrate resulting in half-maximum velocity) and V_{max} (maximum velocity) of aniline hydroxylase and aminopyrine demethylase in hepatic microsomes of guinea pigs, and the mechanisms responsible for the observed effects.

The objective of this study is to ascertain the fate of the oxidative metabolisms of perioperatively administered drugs during isoflurane anaesthesia.

EXPERIMENTAL

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

Reagents and materials

Isoflurane was obtained from Dainabot Co., Japan, and NADPH was purchased from Boehringer Mannheim (Germany). Aniline and authentic formaldehyde were provided by Katayama Chemicals, Japan, and aminopyrine and authentic *p*-aminophenol were obtained from Aldrich Chemical Company, Inc. (USA). The other reagents were commercial products of analytical grade. The butyl rubber cap was purchased from Maruemu (Japan).

Animals and preparation of microsomes

Adult male Hartley guinea pigs, weighing 230–270 g, were purchased from Kyushu Experimental Animals Center (Oita, Japan). Without any pretreatment, the guinea pigs were fasted for 24 h before sacrifice. Each animal was sacrificed by a blow to the head to cause cervical dislocation and immediately thereafter the liver was excised and weighed. After irrigation with ice-cold physiological saline solution through the portal vein to remove blood, the liver was minced with scissors and placed in 0.1 M potassium phosphate buffer (pH 7.4). The minced liver was then homogenized with 0.1 M potassium phosphate buffer (pH 7.4), the volume being more than six times the wet liver weight in a motor-driven Potter homogenizer using a Teflon pestle. All the livers used for each assay group were homogenized under identical conditions with the same homogenizer. The homogenates were then centrifuged in a refrigerated centrifuge at 9000 *g* for 20 min and the post-mitochondrial supernatant was aspirated with care to avoid both the pellet at the bottom and the fatty layer at the top of the centrifuge tube. Microsomal fractions were prepared by centrifuging the 9000 *g* supernatant at 105 000 *g* for 60 min in a Beckman refrigerated ultracentrifuge. The resultant microsomal pellets were then rinsed with 0.1 M potassium phosphate buffer (pH 7.4) to remove cytoplasm. These rinsed microsomal fractions were finally resuspended in 0.1 M buffer (TRIS·HCl: pH 8.0 for aniline hydroxylation and potassium phosphate, and pH 7.4 for aminopyrine N-demethylation) to prepare for the reaction mixture of the assay systems. All the above procedures were carried out at 0–4°C.

Assays of drug metabolism

Oxidative metabolism of aniline and aminopyrine was investigated as hydroxylation and N-demethylation, respectively.

Aniline hydroxylation. After determining the optimal conditions for the assay, a 0.2-ml aliquot of microsomal suspension equivalent to 0.1 g wet liver weight was taken into a 13.5-ml glass test tube and 0.1 ml of aniline was added to the microsomal suspension (final concentration ranged between 0.5 and 30 mM). Then the test tube was sealed with a butyl rubber cap. Isoflurane was injected (concentration in reaction mixture ranged between 0.33 and 3.33 mM) through a microsyringe into the reaction mixture. After pre-incubation at 37°C for 5 min, 0.1 ml of NADPH (1.5 mM) was injected rapidly through the rubber cap to initiate the reaction. The final volume of the reaction mixture was adjusted to 1 ml by adding 0.1 M TRIS·HCl buffer (pH 8.0). Reaction with moderate shaking for 20 min was terminated by mixing 0.5 ml of 20% trichloroacetic acid. Aniline hydroxylation to *p*-aminophenol was determined spectrophotometrically by the method of Imai *et al.*,[11] where the sample without aniline was used as reference and authentic *p*-aminophenol as standard calibrator.

Aminopyrine N-demethylation. After determining the optimal conditions for the assay, the conditions employed were the same as the assay of hydroxylation of aniline except that:

(i) 0.1 ml of aminopyrine was added (final concentration ranged between 0.5 and 20 mM) to 0.2 ml of 0.1 M potassium phosphate buffer (pH 7.4) containing microsomal fractions;
(ii) 0.1 ml of NADPH was rapidly injected through the rubber cap to make the final 3 mM concentration;

(iii) 1 ml total volume was adjusted by means of 0.1 M potassium phosphate buffer (pH 7.4);
(iv) the reaction was terminated by the addition of 1.5 ml of 12.5% trichloroacetic acid and the formed amount of formaldehyde was measured by Nash's method[12] with slight modification, as we used a microplate reader (Model MTP-120, Corona Electronic Co. Ltd. Japan) at wavelength of 415 nm wavelength instead of a spectrophotometer, where the same without aminopyrine was used as reference and authentic formaldehyde to make a standard calibration.

Assay of cytochrome P–450 reduction rate

In an anaerobic cuvette sealed with a rubber cap containing 3 ml of liver microsomal suspension (0.3 g of wet liver weight) in 0.1 M potassium phosphate buffer (pH 7.4), oxygen-free carbon monoxide was bubbled for 5 min. Then 1.48 mM isoflurane was injected except for the control cuvette. A 50-μl aliquot of 0.05 M NADPH was injected through the rubber cap to initiate the reduction. The changes in absorbances at 450 nm were recorded by a Shimadzu UV-300 spectrophotometer to measure the CO–cytochrome P–450 complex formation at various times (P–450v) until the reaction was completed. After removing the rubber cap, a few crystals of sodium dithionite were added to both the experimental and reference cuvette, and the absorbances were measured at both 450 and 490 nm for the estimation of total cytochrome P–450 content (P–450t). The difference between P–450t and P–450v was the unreduced amount of cytochrome P–450. The absorbances of unreduced cytochrome P–450 (P–450t − P–450v) were plotted on semilogarithmic paper. The rate constant of the initial phase (0–3 s), k, was estimated by computer (Macintosh®) using Cricket Graph from the initial phase of the logarithmic plots. The half-time, $t_{1/2}$ (s), for the initial phase of CO–cytochrome P–450 complex formation was calculated from the formula $t_{1/2} = 0.693/k$.[13] The experiments were performed at 25°C.

Assay of protein and cytochrome p-450

The amounts of protein and cytochrome P–450 contained in the microsomal fractions used for both aniline hydroxylation and aminopyrine N-demethylation assays were measured following the methods described by Lowry *et al.*[14] and Omura and Sato,[15] respectively.

Statistical analysis

The formation of *p*-aminophenol and formaldehyde was determined by calculating the obtained absorbances with the calibrations of known concentrations of authentic *p*-aminophenol and formaldehyde, respectively. Then K_m and V_{max} for *p*-aminophenol and formaldehyde formations were calculated by plotting the Lineweaver–Burk plots using a Kaleida® graph (version 2.1.3; Synergy Software, PA, USA) for the Macintosh® (Apple, CA, USA), where 1/substrate concentration was plotted on the *x*-axis and 1/production of metabolites in different concentrations of isoflurane was plotted on the *y*-axis. Mean and standard deviations were computed for all the data in each group. The data were analysed with one-way analysis of variance (ANOVA) followed by a *post hoc* multiple comparison statistical test of Bonferroni/Dunn using the computer program Stat View® 4.0 (Abacus Concepts Inc., CA, USA) for the Macintosh®; $P < 0.05$ was considered to indicate a significant difference.

RESULTS

Effect of isoflurane on aniline hydroxylation

Isoflurane markedly increased the rate of metabolism of aniline to *p*-aminophenol. As can be seen in Table 1, isoflurane concentrations of 0.33–3.33 mM increased the rate of aniline hydroxylation in the guinea pig liver microsomes when compared with the 0 mM isoflurane groups. Maximum enhancement occurred at concentrations of 0.33 and 0.67 mM isoflurane and gradually the enhancing activity faded with increasing concentrations of isoflurane. Note that when the aniline concentration was as much as 30 mM, the enhancing action of isoflurane was negligible.

Effect of isoflurane on aminopyrine N-demethylation

Aerobically formaldehyde was produced by guinea pig liver microsomes through aminopyrine N-demethylation at various concentrations of aminopyrine, and isoflurane depressed the rate of demethylation of aminopyrine in a dose-dependent fashion. Table 2 shows in detail the quantitative inhibition of the aminopyrine demethylase product, formaldehyde, at four different isoflurane concentrations compared with the production in the 0 mM isoflurane groups. The results show that isoflurane decreased the formaldehyde formation as aminopyrine metabolite in a dose-dependent fashion. It is noteworthy that the inhibitory effect of isoflurane is greater at low concentration of aminopyrine than at higher concentration.

Effect of isoflurane on the kinetic properties of aniline hydroxylation and aminopyrine N-demethylation

To determine the kinetic changes of hydroxylation of aniline and of demethylation of aminopyrine, the Michaelis constant K_m (concentration of substrate resulting in half-maximum velocity) and the maximum velocity, V_{max} were calculated by plotting the double reciprocal Lineweaver–Burk plots for the control and each isoflurane concentration group used for both metabolism assays.

Figure 1 summarizes the results of K_m and V_{max} for the aniline hydroxylation reaction. The V_{max} values for aniline hydroxylation were increased significantly with isoflurane compared to the control value, except at higher isoflurane concentration (3.33 mM) where the difference was not significant (Fig. 1a).

In the presence of isoflurane, the K_m values were significantly decreased compared to the control condition. The inhibitory effect on the K_m gradually

Table 1. *p*-Aminophenol formation by aniline hydroxylation in the presence and absence of isoflurane

	Concentration of isoflurane (mM)				
Aniline (mM)	Control (0 mM)	0.33 mM	0.67 mM	1.33 mM	3.33 mM
0.5	0.86 ± 0.20	1.36* ± 0.28	1.33* ± 0.21	1.21* ± 0.23	1.07 ± 0.14
1	1.09 ± 0.21	1.73* ± 0.28	1.73* ± 0.22	1.62* ± 0.22	1.39* ± 0.18
2	1.36 ± 0.22	2.11* ± 0.29	2.16* ± 0.27	1.99* ± 0.24	1.73* ± 0.21
4	1.77 ± 0.24	2.45* ± 0.35	2.53* ± 0.21	2.41* ± 0.21	2.13* ± 0.17
8	2.24 ± 0.27	2.67* ± 0.33	2.83* ± 0.19	2.78* ± 0.22	2.47 ± 0.23
10	2.41 ± 0.3	2.74 ± 0.36	2.90* ± 0.22	2.86* ± 0.20	2.60 ± 0.26
20	2.79 ± 0.32	2.97 ± 0.34	3.03 ± 0.22	3.00 ± 0.23	2.84 ± 0.24
30	2.97 ± 0.34	2.98 ± 0.35	3.11 ± 0.20	3.02 ± 0.22	3.00 ± 0.28

The 1-ml volume reaction mixtures consisted of guinea pig liver microsomes (0.1 g wet liver), aniline (final concentration 0.5–30 mM) and NADPH (final concentration 1.5 mM), with or without isoflurane (0–3.33 mM). After 20 min of incubation at 37°C, the resultant *p*-aminophenol was measured spectrophotometrically. Values are means ± SD ($n = 6$), in which each determinant was replicated twice. * Significantly different ($P < 0.05$) from corresponding values in the control groups.

Table 2. Formaldehyde formation by aminopyrine N-demethylation in the presence and absence of isoflurane

	Concentration of isoflurane (mM)				
Aminopyrine (mM)	Control (0 mM)	0.33 mM	0.67 mM	1.33 mM	3.33 mM
0.5	7.2 ± 1.1	5.2* ± 0.4	4.1* ± 0.3	3.6* ± 0.2	3.0* ± 0.2
1	10.0 ± 1.2	7.5* ± 0.4	6.8* ± 0.2	5.9* ± 0.3	5.0* ± 0.5
2	13.8 ± 1.6	11.2* ± 0.6	10.5* ± 0.7	9.2* ± 0.5	7.8* ± 0.6
5	17.6 ± 1.5	15.8* ± 0.8	15.4* ± 0.8	13.9* ± 0.9	12.3* ± 0.8
10	20.4 ± 1.7	19.2 ± 1.0	18.4* ± 0.8	17.7* ± 1.4	16.0* ± 1.2
15	22.0 ± 2.0	20.6 ± 0.9	20.4 ± 1.5	20.0 ± 1.0	17.6* ± 1.2
20	23.5 ± 2.0	22.2 ± 1.2	21.1* ± 1.4	20.8* ± 0.9	18.6* ± 1.3

The 1-ml volume reaction mixtures consisted of guinea pig liver microsomes (0.1 g wet liver), aminopyrine (final concentration 0.5–20 mM) and NADPH (final concentration 3 mM), with or without isoflurane (0–3.33 mM). After 20 min of incubation at 37°C, the resultant formaldehyde was measured using a microplate reader. Values are means ± SD ($n = 6$), in which each determinant was replicated twice. * Significantly different ($P < 0.05$) from corresponding values in the control groups.

decreases with elevation of isoflurane concentration (Fig. 1b), which consequently supports the result that aniline hydroxylation-enhancing activity gradually becomes lower with increasing concentration of isoflurane. The K_m values of the 0.33, 0.67 and 1.33 mM isoflurane concentration groups are not significantly different from each other, whereas the value for the 3.33 mM concentration group is considerably higher than that for the 0.33 and 0.67 mM concentration groups. These results are suggestive of peak stimulation by isoflurane between 0.33 and 0.67 mM concentrations. This finding indicates stimulation of enzyme activity by isoflurane.

For aminopyrine N-demethylation, K_m and V_{max} values are shown in Fig. 2. Figure 2a illustrates that at higher isoflurane concentration (3.33 mM) the V_{max} value was significantly lower than that of the control, although there was a tendency for V_{max} to decrease among the other isoflurane concentration groups.

Isoflurane caused a significant increase in the K_m values for formaldehyde formation when compared with that in the control group, and the K_m values of each isoflurane concentration group increased markedly in a dose-dependent fashion except for the 3.33 mM isoflurane concentration group, which was not significantly different from the 1.33 mM concentration group (Fig. 2b). This result indicates a dose-dependent inhibitory effect of isoflurane on aminopyrine N-demethylation.

The units were expressed in both assays as nmol·nmol· P–450^{-1}· min^{-1} and μM for V_{max} and K_m, respectively. The protein content of microsomes was measured and cytochrome P–450/protein ratios (mean ± SD) were 0.39 ± 0.03 nmol P–450 mg^{-1} protein for the aniline assay and 0.42 ± 0.05 nmol P–450 mg^{-1} protein for the aminopyrine assay.

Effect of isoflurane on the cytochrome P–450 reduction rate

The rate of cytochrome P–450 reduction by NADPH was determined by estimating the carbon monoxide–cytochrome P–450 complex formed. The half-time for the formation of the carbon monoxide–cytochrome P–450 complex was shortened significantly by 44% of the control value in the presence of 1.48 mM isoflurane (Fig. 3), which is suggestive of an increased cytochrome P–450 reduction rate in the presence of isoflurane.

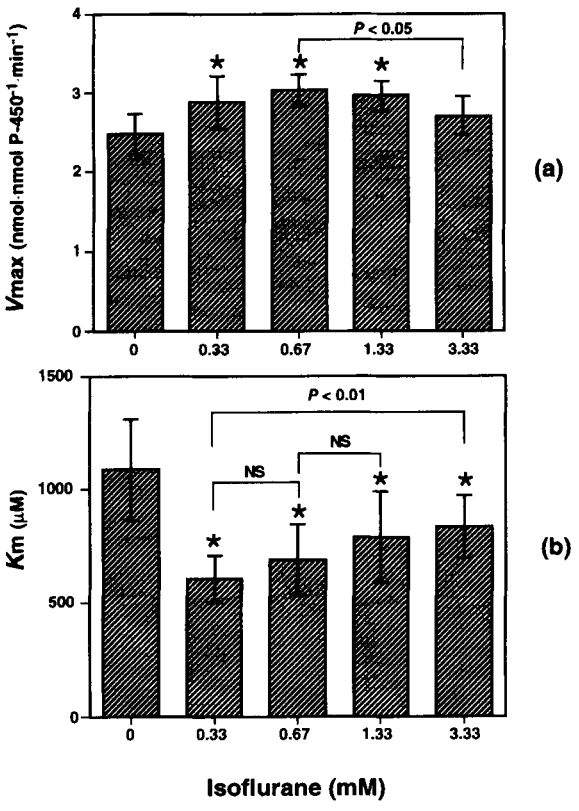

Figure 1. The effect of isoflurane on the kinetic properties of aniline hydroxylation. **(a)** Effect on V_{max} for *p*-aminophenol formation. **(b)** Effect on K_m for *p*-aminophenol formation. Determination of V_{max} and K_m for *p*-aminophenol formation is described in the Experimental section. Each column in both graphs represents the mean ± SD ($n = 6$), in which each determinant was replicated twice. The *x*-axis of each graph shows anaesthetic concentration in the reaction mixture. * Significantly ($P < 0.05$) different from corresponding value in the control group. NS indicates that difference is not significant.

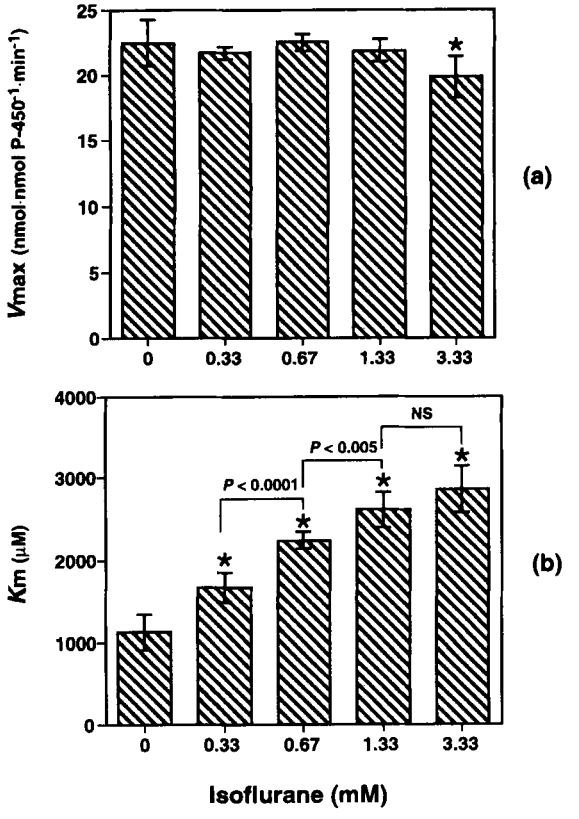

Figure 2. The effect of isoflurane on the kinetic properties of aminopyrine N-demethylation. **(a)** Effect on V_{max} for formaldehyde formation. **(b)** Effect on K_m for formaldehyde formation. Determination of V_{max} and K_m for formaldehyde formation is described in the Experimental section. Each column in both graphs represents the mean ± SD ($n = 6$), in which each determinant was replicated twice. The *x*-axis of each graph shows anaesthetic concentration in the reaction mixture. * Significantly ($P < 0.05$) different from corresponding value in the control group. NS indicates that difference is not significant.

DISCUSSION

Aminopyrine and aniline were selected as substrates for this study on the basis of their previously known characteristics as described by Schenkman *et al.*[16] Generally the drugs are classified into three types according to their distinct type of difference spectrum when combined with hepatic microsomes. These changes are believed to represent the binding of drugs with the terminal oxidase of the hepatic microsomal mixed-function oxidase system, cytochrome P–450. Type I drugs (such as aminopyrine) have a maximum absorption peak at 385–390 nm and a minimum at about 420 nm wavelength. Almost symmetrical to type I, type II drugs (such as aniline) have a maximum absorption peak at about 430 nm and a minimum at 390 nm wavelength. Certain compounds such as acetanilide show a difference spectrum slightly different from type II and are classified as modified type II drugs.

It is interesting that isoflurane influenced the metabolism of type I and type II drugs in a contrasting manner, although isoflurane itself is only 0.2% metabolized. Isoflurane, being a type I drug, caused a dose-dependent competitive inhibition of type I drug aminopyrine metabolism, but enhanced the metabolism of the type II drug aniline in our study (Tables I and 2).

Alteration of the hepatic microsomal metabolism of drugs *in vitro* has been reported by several investigators, and some are described in the experimental section. Volatile anaesthetics do not act as inducers of enzymes that carry out hepatic microsomal biodegradation. This could be presumed by the contrasting effect of isoflurane on aniline and aminopyrine breakdown, since both of the above biodegradations involve hepatic cytochrome P–450 enzyme as the terminal oxidase. In 1973, Korten and Van Dyke reported that halothane, methoxyflurane and chloroform in low and high concentrations stimulated enzyme activity during aniline hydroxylation, while the same anaesthetics inhibited the aminopyrine N-demethylation reaction except with a low concentration of halothane.[17] In the same study, inhibition of aniline as well as aminopyrine biodegradation by diethyl ether was also observed. It is a very complicated mechanism by which different volatile anaesthetics act differently on metabolism.

To elucidate the mechanism responsible for alteration of metabolism, we must go through the microsomal mixed-function oxidase system, since metabolism of most of the drug occurs through this system. The microsomal mixed-function oxidase system consists of

several steps, where the terminal enzyme is cytochrome P–450. The data are not presented here but we observed in a previous study that isoflurane did not have any effect on NADPH–cytochrome P–450 reductase, which is the initial one among the several steps. During this study it was observed that isoflurane caused activation of the rate of cytochrome P–450 reduction by NADPH, as could be presumed by the decrease of the half-time ($t_{1/2}$) for the formation of carbon monoxide–cytochrome P–450 complex in the presence of isoflurane (Fig. 3). This finding confirms the report of Gigon *et al.*,[18] who suggested that substrates which induce type I spectral changes of cytochrome P–450 increased the rate of its reduction by NADPH and, as in our study, isoflurane is a type I substrate.

According to Yoshida and Kumaoka, type I drugs combine with the protein part of cytochrome P–450, whereas type II drugs combine with the haeme part of the same.[18] During aminopyrine N-demethylation we used aminopyrine and isoflurane, both of which are type I drugs. Thus, aminopyrine and isoflurane both combine with cytochrome P–450 at the same protein part. Although we have shown that isoflurane activated the rate of cytochrome P–450 reduction by NADPH, the total formaldehyde formation from aminopyrine demethylation was decreased by the influence of isoflurane. The reason for this effect is that the competitive action of aminopyrine and isoflurane for the same binding site might be much stronger than the stimulatory effect of isoflurane on the rate of cytochrome P–450 reduction by NADPH. If the binding sites of aminopyrine and isoflurane with cytochrome P–450 are different from each other, the formation of formaldehyde should increase due to the stimulatory effect of isoflurane on the cytochrome P–450 reduction rate. However, our data showed that the total formaldehyde formation decreased significantly with isoflurane as a result of competition between the two drugs for the same binding site during metabolism. This finding consequently proves that the competitive effect is greater than the stimulation of cytochrome P–450 reduction with isoflurane. In Fig. 2b, the K_m values for aminopyrine demethylation indicate that the competitive inhibitory effect of isoflurane was dose dependent, suggesting that a higher isoflurane concentration leaves less binding sites for aminopyrine. In Fig. 2a, the V_{max} value for formaldehyde formation at 3.33 mM isoflurane concentration was significantly lower than the control group. On the other hand, at the same anaesthetic concentration, the K_m value (Fig. 2b) was not significantly different from the K_m value of the nearest 1.33 mM concentration of isoflurane. This effect is due to saturated cytochrome P–450 and isoflurane binding activity.

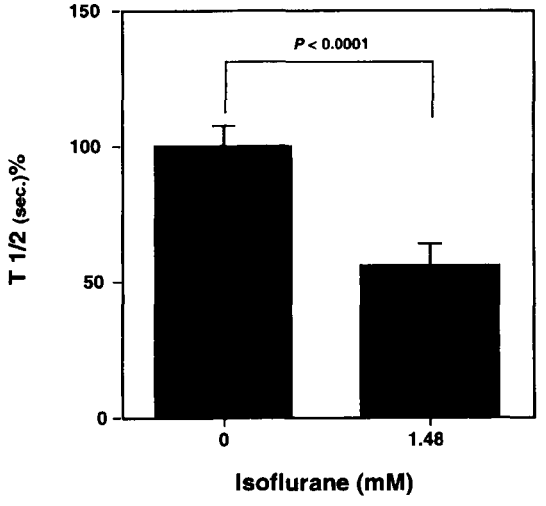

Figure 3. The effect of isoflurane on CO–cytochrome P-450 complex formation was measured spectrometrically. Conditions for the reaction mixture and determination of half-time, $t_{1/2}$ (s), for the initial phase of CO–cytochrome P-450 complex formation are described in detail in the Experimental section. The $t_{1/2}$ (s) value of the control group was taken as 100% and that for the 1.48 mM isoflurane concentration (anaesthetic concentration in reaction mixture) group was converted to a percentage. Each column represents the mean ± SD ($n = 5$).

Isoflurane increased the rate of metabolism of aniline; aniline is a type II drug and hence it combines with the haeme iron of the cytochrome P–450 enzyme. Here, the type I drug isoflurane has a binding site different from aniline and as a result of the stimulatory effect of isoflurane on the rate of cytochrome P–450 reduction by NADPH, which is one of several steps of the microsomal mixed-function oxidase system, the formation of *p*-aminophenol from aniline hydroxylation was increased significantly in the presence of isoflurane. It has also been reported that type II substrates inhibit the rate of cytochrome P–450 reduction by NADPH.[19] In view of their report, we can assume from our results that the stimulatory effect of isoflurane on the reduction rate of cytochrome P–450 is greater than the inhibitory effect of aniline on the same, and thus the total effect, that is total *p*-aminophenol formation, was increased in the presence of isoflurane. However, our speculation based on the present findings differs from the previous report of Yoshida and Kumaoka, because our experimental method of cytochrome P–450 reduction by NADPH was similar to theirs. We suggest that the decreased CO–cytochrome P–450 complex formation in the presence of type II substrate was not due to the effect on the cytochrome P–450 reduction rate, rather it was a competition of type II substrate and CO for the same binding site, since type II substrate and CO (modified type II) both combine at the haeme part of cytochrome P–450. Accordingly, we can simply explain in the present study that the stimulatory effect of isoflurane (type I) on the reduction rate of cytochrome P–450 is the most reliable cause of the increased aniline hydroxylation by isoflurane, since aniline has a different enzyme binding site from that of isoflurane.

Unusual kinetic properties were observed during anilinchhydroxylation in the presence of isoflurane. At low isoflurane concentrations the K_m values for aniline hydroxylation were decreased, but the value was elevated at higher concentration, even though the elevated value was significantly lower than the control group (Fig. 1b) and the V_{max} value at the same higher concentration decreased when compared with the corresponding values of other low concentration groups (Fig. 1a). This result indicates that activation of the reaction by isoflurane peaked at low anaesthetic concentrations because the K_m values of low concentration groups

(0.33, 0.67 and 1.33 mM) were not significantly different from each other, but the activation became lower at higher concentrations of isoflurane. This might be due to some inhibitory effect of excess isoflurane on the mixed-function oxidase system after the step of cytochrome P–450 reduction by NADPH, or excess isoflurane binding with the hydrophobic protein part of cytochrome P–450 might cause structural changes of that part which resulted in decreased enzyme activity. Further investigation on this inhibitory effect of excess isoflurane is mandatory.

Although no complete clinical interpretation of the data presented here can be made now, being an animal *in vitro* study, it is of interest to speculate that the rate of metabolism of various drugs used during the course of isoflurane anaesthesia may change. This evidence also strongly supports the evidence presented elsewhere with other anaesthetics, but it should be cautioned that the presence of isoflurane may alter the reactions, since different types of samples are taken from anaesthetized animals and humans for *in vitro* studies.

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