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EFFECT OF OXYGEN CONCENTRATION ON THE REACTION OF HALOTHANE WITH CYTOCHROME P450 IN LIVER MICROSOMES AND ISOLATED PERFUSED RAT LIVER

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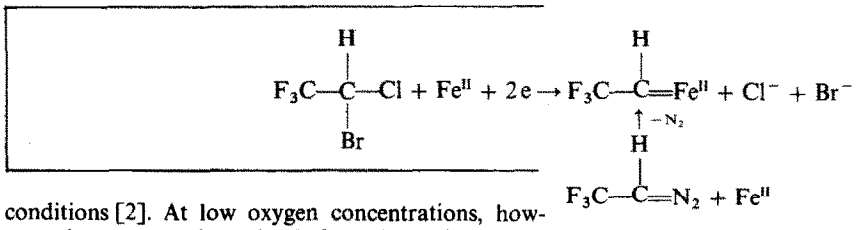
Abstract—The influence of oxygen on the complex formation of reduced cytochrome P450 with halothane has been investigated with liver microsomes and perfused livers from phenobarbital-pretreated rats. The reductive formation of the trifluoro carbene complex from halothane in liver microsomes was inhibited at high oxygen concentrations but started to appear below 50 μM oxygen and was maximal under anaerobic conditions. Metyrapone was an efficient inhibitor of the carbene complex formation. Organ spectrophotometry of isolated perfused livers established that the complex appeared already under slightly hypoxic conditions and that metyrapone addition to the perfusion medium abolished its formation. The results indicate the possibility of a reductive *in vivo*-metabolism of halothane to reactive intermediates when the oxygen concentration of the cell becomes lower than about 50 μM.

The widely used anaesthetic halothane (2-bromo-2-chloro-1.1.1.-trifluoroethane) is metabolized to a considerable extent in man and trifluoroacetic acid is found as the main metabolite *in vivo* [1] and *in vitro* [2]. This reaction occurs under high oxygen concentrations and must be regarded as a real detoxication pathway since covalent binding of ^{14}C-labeled halothane to tissue components is low under these conditions [2]. At low oxygen concentrations, however, the amount of covalently bound metabolites increases which suggests the involvement of a reductive rather than an oxidative pathway in covalent binding [3, 4]. The question of the underlying mechanism is of importance, since a correlation could exist between the formation of reactive intermediates and some reports on hepatotoxic effects after halothane anaesthesia [5–7].

Using inducers and inhibitors of the liver microsomal drug monooxygenase system it was shown that cytochrome P450 catalysed the oxidative as well as the reductive pathway of halothane [2, 3]. It was of particular interest that reduced cytochrome P450 formed a complex with halothane which exhibited an unusual Soret band at 470 nm [4]. On the basis of a chemical study we have proposed that this spectrum represents a complex between the ferrous cytochrome P450 and trifluoromethyl carbene formed by reductive elimination of chloride and bromide from the halothane molecule according to the following reaction [8]:



2.2.2-trifluorodiazoethane, which readily undergoes a metal-catalyzed decomposition to dinitrogen and this carbene, forms an identical difference spectrum with liver microsomal cytochrome P450.

Such a carbene species when liberated from the heme must be regarded as a highly reactive intermediate but it remained to be shown that it could be formed under *in vivo* conditions when oxygen was present. Since reduced cytochrome P450 has a high affinity towards its physiological substrate oxygen it could be expected that the formation of the carbene complex would be limited to anaerobic conditions. In this paper we report on the effect of oxygen concentration on the reductive metabolism of halothane in liver microsomes and isolated perfused liver.

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MATERIALS AND METHODS

A. Liver microsomes. Male Sprague–Dawley rats (100–150 g) were pretreated for three days by a daily injection (i.p.) of 80 mg/kg body wt. of phenobarbital. Liver microsomes were prepared as previously described [9]. Protein was determined by the biuret method of Gornall *et al.* [10].

Difference spectra of microsomal suspensions were recorded on an Aminco DW-2 spectrophotometer using 10 mm cuvettes. The stock solution of microsomes was diluted in 0.1 M Tris–HCl buffer pH 7.6 to 6.0 ml and equally divided into two cuvettes. After recording the baseline, μl-additions of the compounds (dissolved in methanol) were made and corresponding amounts of solvent were added to the reference cell. For simultaneous recording of the oxygen concentration a rotating and stirring oxygen electrode was built and mounted on top of the cuvette.

B. Perfused livers. Livers from male Wistar rats weighing 150–180 g, fed on stock diet (Altromin®) and pretreated with phenobarbital for 3 days prior to the experiment (80 mg/kg i.p.), were perfused as described previously [11] except that L-lactate in the perfusate was 2.1 mM. Perfusate flow was 3.5–4.0 ml/min; the temperature was 36.5–37°. Halothane was infused from a stock solution of 0.3 or 0.1 M in methanol, to give a final concentration in the perfusate of about 0.21 mM.

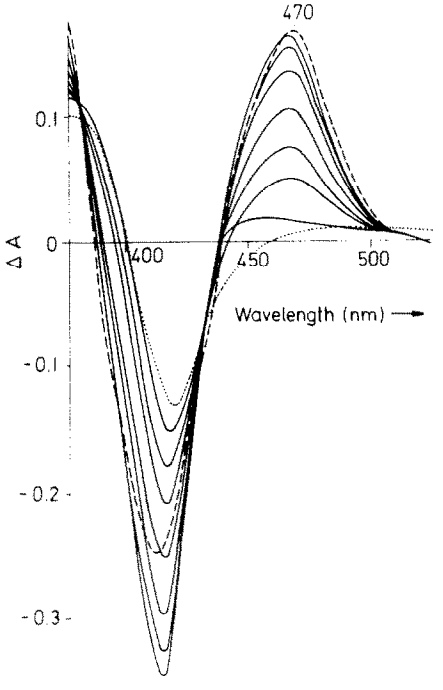

Fig. 1. Formation of the 470 nm absorption band in anaerobic rat liver microsomes in the presence of halothane and NADPH. The two cuvettes contained 3 ml of a microsomal suspension of 7.5 mg of protein/ml (2.8 nmoles cytochrome P450/mg protein). Halothane (1 mM) was added to the sample cuvette (dotted line). 100 μl of a 10^{-2} M solution of NADPH were added to both cuvettes and the difference spectra at 0.5, 6.0, 6.5, 7, 7.5, 8, 12 and 16 min recorded (solid line). At the end of the experiment about 2 mg of sodium dithionite were added to both cuvettes (dashed line).

Organ spectrophotometry was performed with a special photometer (designed and constructed in the Electronics Department of "Sonderforschungsbereich Medizinische Molekularbiologie und Biochemie", Munich [12]). Spectra, processed with a Nicolet Model 1072 instrument computer, were obtained by the following procedure: a reference spectrum was stored in one half of the computer memory (12 bit resolution), and then the experimental change (e.g. halothane infusion) was made. A new spectrum was recorded, and after ascertaining that a stationary state existed, was stored in the other half of the memory. The difference spectrum was obtained by digital subtraction of the reference spectrum and plotted by an XY-recorder. Dual-wavelength photometry through a liver lobe was performed as previously described [12].

Using a gas-mixing device based on Rotameter flow meters, the proportion of oxygen, nitrogen and carbon monoxide (all three containing 5% CO_2) in the gas mixture was controlled. Hypoxia was monitored by measurement of cytochrome oxidase (445–460 nm) absorbance. Zero per cent reduction was taken as the steady state in O_2, 100 per cent reduction was taken as the steady state in N_2.

RESULTS

When halothane was added to liver microsomes from phenobarbital-pretreated rats a substrate binding difference spectrum appeared, which showed saturation above a concentration of 1 mM halothane. Addition of NADPH to both cuvettes caused a rather slow formation of an absorption band around 470 nm which was fully developed after 10 min and did not further increase after addition of sodium dithionite (Fig. 1).

An initial lag phase indicated that residual oxygen was removed by the system prior to reduction of halothane, yielding the trifluoromethyl carbene complex at cytochrome P450. The competition between oxygen and halothane reduction can be demonstrated by a simultaneous recording of oxygen concentration and absorbance difference Δ A (470–490 nm) in a closed cuvette (Fig. 2).

Only when oxygen was largely removed from the solution did the Δ A (470–490 nm) begin to rise. Readmission of oxygen caused a slow and partial reversal, whereas dithionite addition brought the oxygen concentration to zero and Δ A (470–490 nm) to its maximum value.

The critical oxygen concentration at which formation of the carbene complex is first observed was of particular interest. An experiment similar to the one shown in Fig. 2 was carried out at an expanded scale and showed that the formation of the carbene complex starts at about 33 μM oxygen.

In other experiments this number varied between 20 and 60 μM and an average value of about 34 μM oxygen was calculated.

The kinetics of the anaerobic carbene complex formation are rather slow with a time of half maximal formation of about 2 min. Hence it could be argued that the consumption of oxygen in these experiments occurred at a faster rate than the complex formation so that no equilibrium conditions were obtained. In order to measure the complex formation under

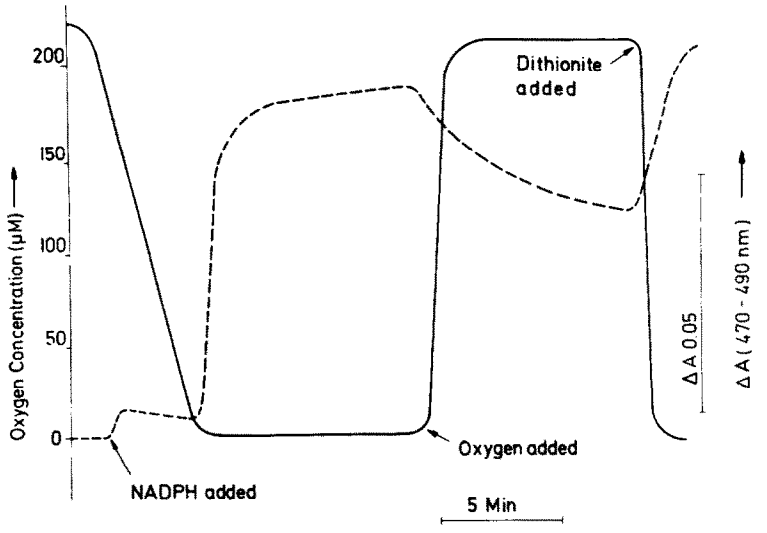

Fig. 2. Simultaneous recording of oxygen concentration (solid line) and △ A(470–490 nm) absorbance difference (dashed line) of liver microsomes in the presence of halothane (1 mM) and NADPH (1 mM). A special cuvette with a rotating clark-type oxygen electrode was placed in an Aminco DW-2 spectrophotometer and the absorbance difference between 470 and 490 nm was monitored. The cuvette contained 7.5 mg of microsomal protein (2 nmoles cytochrome P450/mg protein).

defined equilibrium conditions of oxygen pressure, microsomal suspensions were gassed with O_2-N_2 mixtures and the corresponding steady state concentration of the 470 nm complex were followed in the presence of the halogenated ethane and NADPH (Fig. 3).

Under these conditions the reductive complex formation started already at higher oxygen concentrations and reached half-maximal saturation at about 40 μM. The reproducibility of the values was rather poor, since the system continuously consumed oxygen and especially at lower oxygen concentrations and partial pressure could not be kept constant over the time of the experiment. It was noted that the absorbance at 470 nm started to rise suddenly and then slowly proceeded to its final value. As already indicated in Fig. 2 an excess of oxygen did not fully reverse the complex formation, so that only a partially reversible reaction is observed. Nevertheless, it can be concluded that the carbene complex formation starts already at an oxygen concentration above 50 μM.

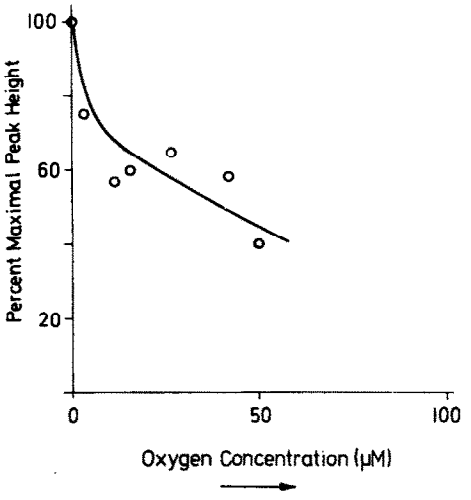

Fig. 3. Reductive complex formation of halothane with reduced cytochrome P450 under steady-state oxygen concentrations. The experiments were performed in a closed cuvette with gas inlet and outlet and a Clark-type oxygen electrode (see Fig. 2). The microsomal suspension (3.0 ml containing 7.5 mg protein and 2.2 nmoles cytochrome P450/mg protein) was gassed with defined O_2-N_2 gas mixtures for 2 min and then halothane (1 mM) and NADPH (1 mM) were added. The peak formation was monitored at 470–490 nm under continuous recording of the oxygen pressure. Maximum complex formation was obtained after addition of sodium dithionite (2 mg in 10 μl of water).

The interaction of halothane with cytochrome P450 was further investigated in the isolated perfused rat liver. The application of special organ-spectrophotometric techniques had earlier proved suitable for demonstrating the interaction of substrates and ligands of cytochrome P450 as well as changes in the redox state of the cytochrome during monooxygenation [13]. Difference spectra from a lobe of perfused liver from a rat pretreated with phenobarbital are shown in Fig. 4.

The difference spectrum of curve A was obtained when the spectrum of an anaerobic liver lobe was subtracted from the corresponding spectrum in the presence of carbon monoxide. The absorbance at 450 nm represents the carbon monoxide complex of fully reduced cytochrome P450. The absorbance change observed upon infusion of 0.21 mM halothane (in the absence of carbon monoxide) is shown in curve B. It exhibits a peak at about 470 nm, resembling the halothane difference spectrum found with isolated liver microsomes; a control infusion of solvent (methanol) did not lead to detectable absorbance changes in this spectral region, whereas it did at the wavelengths characteristic of catalase compound I at around 405 nm and 660 nm [16]. Similarly, there was

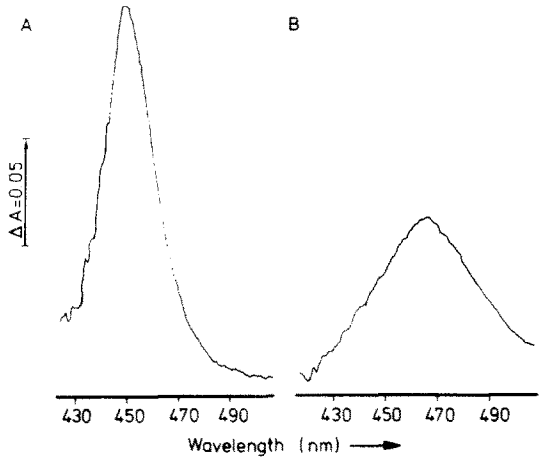

Fig. 4. Difference spectra from a lobe of perfused liver from a rat pretreated with phenobarbital. Curve A represents the CO-difference spectrum under anoxic conditions. Curve A shows the halothane (0.21 mM) induced difference spectrum under anoxic conditions (in the absence of CO).

no appreciable change of O_2 uptake upon methanol infusion up to 20 mM.

The peak at around 470 nm was formed not only under anoxic conditions but also under slightly hypoxic conditions (Table 1) which were obtained by admission of nitrogen into the gas mixture equilibrating with the perfusate. Under these conditions cytochrome oxidase was reduced 5 per cent compared to 100 per cent reduction in anoxia. By comparing the relative absorbances of the carbon monoxide and the halothane complexes of cytochrome P450 with the maximum peak heights obtained from the spectra of Fig. 5 it can be calculated that about 35 per cent of the maximum halothane induced complex was formed under the slightly hypoxic conditions in the experiment described in Table 1.

At present, the oxygen concentration at the site of cytochrome oxidase cannot be determined directly by available methods. According to the recent discussion by Nicolls and Chance [14] the K_m value for oxygen at cytochrome oxidase may be around 1 μM. If so, the probable oxygen concentrations in the experiment presented in the Table was of the order of 10 μM at the site of cytochrome oxidase in the liver cells. However, the existence of steep O_2 gradients in the intact organ during steady states of hypoxia within the liver lobule [15] may lead to a non-homogeneous distribution of O_2. Thus, a small proportion of liver cells located in the pericentral region of the lobule may be fully anoxic whereas the more periportally located cells may be well-supplied with O_2 under the hypoxic condition present in the experiment of the table. A similar condition may arise also during hypoxic intervals in anaesthesia.

Since the carbene is regarded as a ligand in the 470 nm absorbing cytochrome P450 complex, other known ligands of this cytochrome should be able to compete for the binding site. This can be demonstrated when the carbene complex is formed in the presence of rather low concentrations of halothane in a microsomal suspension in the presence of $Na_2S_2O_4$ and then exposed to increasing concentrations of metyrapone (2-methyl-1,2-bis-(3-pyridyl-1-propanone) (Fig. 5).

Increasing concentrations of this pyridine derivative decrease the 470 nm absorption with a concomitant formation of a 446 nm band characteristic for the cytochrome P450-metyrapone complex. Although this complex has a very low dissociation constant of 1.9 μM [17], complete displacement of the carbene was not achieved.

When metyrapone and halothane are added together to an anaerobic microsomal suspension in the presence of NADPH, the rate of the reductive formation of the carbene complex is only about 1 per cent compared to the rate in absence of metyrapone.

About 25 per cent of the maximum absorption at 470 nm is reached only after 3 hr and it does not increase further.

The blocking effect of metyrapone on halothane reduction was also established in the perfused liver (Fig. 6).

Curve A represents the difference spectrum of the liver lobe under slightly hypoxic conditions. The increase of the degree of reduction of cytochrome oxidase upon addition of halothane under hypoxic con-

Table 1. Appearance of the halothane-dependent absorbance at 469 nm in perfused liver from a phenobarbital-pretreated rat under slightly hypoxic conditions

Component measured (wavelength pair)	Perfusion conditions	Absorbance difference	Remarks
Halothane complex (469–484)	Halothane (0.21 mM) at hypoxia (N_2/O_2, 20/75)	0.028	Reference: Hypoxia, cytochrome oxidase about 5% reduced
Cytochrome oxidase (445–460)	N_2	0.190	Reference: O_2
Cytochrome P450 (450–465)	CO	0.240	Reference: N_2

The perfusion medium contained, in condition (1) 0.21 mM halothane and was gassed with a N_2/O_2 mixture (20/75, v/v with 5 vols % CO_2). Under these conditions, cytochrome oxidase was reduced 5% as compared to the anaerobic state. In conditions (2) and (3), halothane was absent, and a measure of total cytochrome oxidase was obtained from the absorbance difference 445–460 nm in anaerobic and aerobic conditions, whereas a measure of cytochrome P450 was obtained from the absorbance difference 450–465 nm in the presence and absence of CO under anaerobic conditions, respectively.

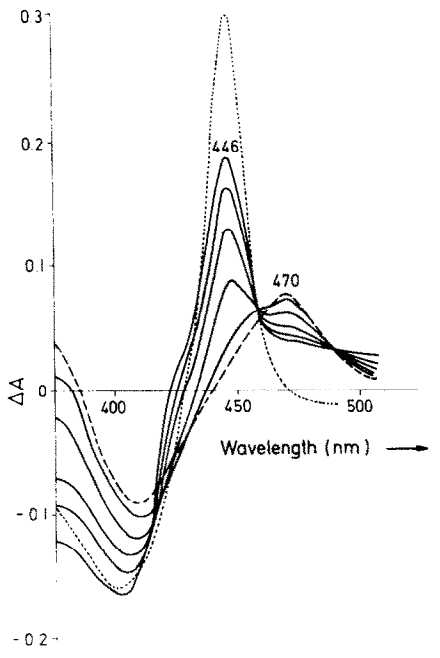

Fig. 5. Effects of increasing concentrations of metyrapone on the halothane induced spectrum of $Na_2S_2O_4$ reduced rat liver microsomes. Each cuvette contained 7.5 mg of microsomal protein (2 nmoles cytochrome P450/mg protein). The dashed curve corresponds to the difference spectrum after addition of 10 μM halothane. The solid lines are the difference spectra after subsequent additions of 0.3, 2, 7, 25 and 100 μM metyrapone. The dotted line was obtained after addition of 100 μM metyrapone to a microsomal suspension of the same concentration without halothane.

ditions (Fig. 6A, peak at 445 nm) is probably due to the extra oxygen uptake caused by halothane, amounting to about 0.4 μmole extra O_2 taken up per min per gram of liver upon addition of 0.2 mM halothane [18].

Addition of a low concentration of metyrapone (0.07 mM) was subsequently performed under anoxic conditions (Fig. 6B). Analogous to the effects observed with microsomes under similar conditions this difference spectrum shows the decrease of the halothane-induced peak concomitant with the formation of the metyrapone complex with reduced cytochrome P450 at 446 nm.

DISCUSSION

The results allow the conclusion that at an oxygen concentration of about 50 μM in the liver cell the reduction of halothane at cytochrome P450 starts to compete with the binding and activation of molecular oxygen by this cytochrome. Although it is difficult to define oxygen concentrations for the liver cell *in vivo*, it can be assumed that such values may occur, especially in the center of the lobules. An oxygen deficiency could be enforced during surgical interventions and could lead to an increase in the reductive metabolism of halothane. Indicative for a reductive pathway is the spectral appearance of the trifluorocarbene complex as previously described. The complex itself is stable under anaerobic conditions but with oxygen present it largely decomposes since it is well-known that carbenes can only be stabilized by the low valence states of iron and other metals. Due to its high reactivity the free carbene will react with components of the system and could lead to covalent binding to proteins or lipids. During this process a release of fluoride could also occur from either the carbene or its secondary reaction products, which would explain the results in literature on fluoride liberation from halothane *in vitro* [19] and *in vivo* [20].

Our results do not exclude other reactive intermediates than the carbene as a consequence of a reductive pathway of halothane. Of main importance with regard to the covalent binding of halothane metabolites to tissue components is the fact that a reductive rather than an oxidative pathway must be involved and that the onset of halothane reduction by cytochrome P450 occurs already at rather high oxygen concentrations. This indicates that reduction

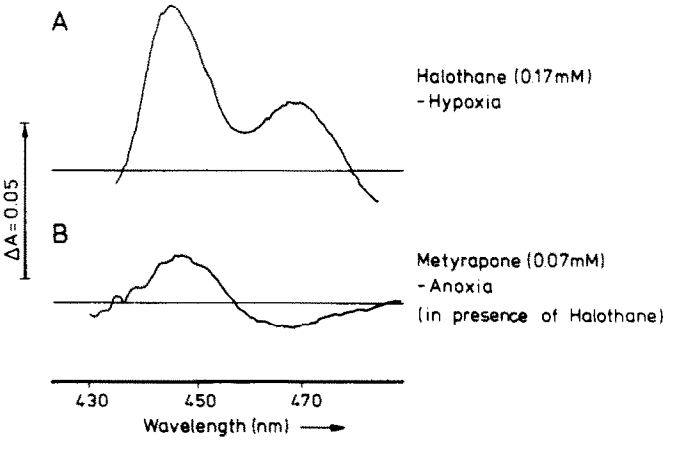

Fig. 6. Effect of metyrapone on the formation of the halothane induced difference spectrum from a lobe of perfused liver from a phenobarbital pretreated rat. The same conditions as in Fig. 4 were used. Difference spectrum A shows the formation of the halothane (0.17 mM) induced peak at 469 nm under hypoxic conditions. Subsequently addition of metyrapone (0.07 mM) gives difference spectrum B under anoxic conditions.

of halothane in the liver cell, especially in the center of the lobules, may not be an unusual event during anaesthesia. The extent, however, will largely depend on the oxygen concentration and will increase exponentially with decreasing oxygen supply.

No direct correlation has yet been shown to exist between the covalent binding and the rare cases of halothane-mediated liver cell necrosis in man. However, an animal model reported recently strongly indicates that this correlation exists [21].

It is of interest to define the conditions that favor the carbene complex formation besides the oxygen concentration. A second parameter is the pattern of cytochrome P450 enzymes that are present in the liver. Several species can be detected in untreated rats and pretreatment by inducers like phenobarbital or 3,4-benzpyrene leads to a considerable increase of only some forms of cytochrome P450 [22]. After pretreatment with phenobarbital [23] or Aroclor 1254 (polychlorinated biphenyls) [21] the covalent binding and the hepatotoxicity increases. After phenobarbital pretreatment of various species the carbene complex formation from halothane was also enhanced [8] and concomitantly was the relative inhibition of drug metabolism by metyrapone [24]. This explains why metyrapone was an effective inhibitor of halothane reduction in our experiments. It seems therefore that not all cytochrome P450 species can bind halothane equally well, which is known also for other substrates. Since we have evidence from liver biopsy studies that in man the pattern of cytochrome P450 and also its concentration shows a large individual variation (P. Stenger, V. Ullrich, unpublished), this may be an important factor for explaining the individual differences in drug metabolism and also for the toxicity of halothane.

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