Kinetics of Methoxyflurane Biotransformation with Reference to Substrate Inhibition

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The kinetics of biotransformation of methoxyflurane by rat hepatic microsomes in vitro was studied. The rate of biotransformation as measured by analysis of metabolites continued to increase even at near-saturation concentrations of the anesthetic. Methoxyflurane biotransformation followed either an unbounded curve with empirical formula \( y = a \ln(x + b) \) or an asymptotic curve with formula \( y = \frac{x}{3x + b} \). No substrate inhibition was observed.

Total fluoride \( V_{max} \) of 135.1 mmol F⁻/mg protein/30 min increased to 391.9 by phenobarbital induction; free fluoride \( V_{max} \) from 39.2 to 401.2. Thus, enzyme induction shifted biotransformation to the production of greater amounts of inorganic free fluoride metabolites than organic fluoride-containing metabolites. Phenobarbital induction caused qualitative as well as quantitative alteration in the biotransformation of methoxyflurane. (Key words: Anesthetics, volatile, methoxyflurane; Biotransformation (drug), methoxyflurane: Induction, enzyme.)

In common with many nonvolatile "fixed" drugs, inhalation anesthetics are recognized as being oxidatively biotransformed to a considerable extent, primarily by the NADPH-dependent mixed-function oxidase system of the hepatic endoplasmic reticulum. It is well established that potent inhalation anesthetics, compounds that have high lipid solubilities, concentrate in fatty tissues. This is particularly true for methoxyflurane, which has the highest fat solubility of any clinically employed inhalation anesthetic. The terminal oxidase of the mixed-function oxidase system, cytochrome P-450, is enmeshed in a phospholipid matrix. The activity of the system is determined by the integrity of this lipid structural relationship, since removal of the matrix by lipid solvents inactivates cytochrome P-450. As the concentration of a lipid-soluble compound increases in this matrix, there would be expected to be inhibition of the activity of the enzymes due to structural alterations. Sawyer et al. suggested that the fraction of anesthetic metabolized by the liver in miniature swine decreased at higher inspired concentrations of the anesthetic on the basis that less anesthetic was removed by the liver at higher concentrations. Although these investigators did not determine that high concentrations of an inhalation anesthetic actually inhibit its own biotransformation, there have been allusions to such substrate inhibition.

The present study was designed to answer three questions: 1) what are the kinetics of methoxyflurane biotransformation; 2) does substrate inhibition occur at high anesthetic concentrations; 3) are qualitative as well as quantitative alterations of methoxyflurane biotransformation produced by induction of hepatic microsomal enzymes by phenobarbital pretreatment? This study employed hepatic microsomal enzymes in vitro so that variables such as hepatic blood flow, differential lipid solubilities, biliary excretion, and total-body equilibration of anesthetic concentrations of intracellular components would be eliminated. Products of metabolism were used as end-points. Methoxyflurane was chosen for study for two reasons. First, it has the highest lipid partition coefficient of the clinically employed anesthetics and thus would be highly likely to inhibit the lipid-enmeshed cytochrome P-450 enzyme system. Second, methoxyflurane has two distinct pathways of biotransformation that can be measured to reveal qualitative differences in metabolism.

Methods

Sixteen Sprague-Dawley and 16 Fischer 344 young adult male rats (250–300 g) were
used. The animals were housed in air-conditioned rooms and had no contact with environmental enzyme-inducing agents such as insecticides, bedding, etc. Eight animals of each strain were pretreated for ten days with phenobarbital (1 mg/ml drinking water) to induce enzymes of the hepatic microsomal electron-transfer chain. Previous studies in this laboratory have demonstrated that such pre-treatment increases cytochrome P-450 content from a control value of 0.635 to 1.27 μmol/mg protein, and NADPH cytochrome c reductase content from 76.7 to 157.7 μmol cytochrome c reduced/mg/min. Animals were sacrificed by cervical dislocation, the livers quickly excised, and the portal veins flushed with iced saline solution. The livers were then minced and homogenized at 2°C with a Teflon pestle for grinding tissue.

The homogenate was centrifuged at 9,500 x g for 30 min and the supernatant removed. The supernatant was then centrifuged in an I.E.C. ultracentrifuge for one hour and the microsomal pellet resuspended in 50 mM Tris-HCl buffer (pH 7.4) to a total protein concentration of 12 mg/ml as determined by the biuret reaction. An NADPH-generating system composed of NADP (2 μmol), glucose-6-phosphate (5 μmol), and glucose-6-phosphate dehydrogenase (1 I.E.U.) was employed. EDTA was added to a final concentration of 0.6 mM. Methoxyflurane was dissolved in buffer at 20°C and aliquots of this added to the microsomal suspensions so that final anesthetic concentrations ranged from 0.06 to 2.5 mM, the latter being nearly saturated. The final volume of each of these test solutions was 3 ml.

Polyethylene vials used for these studies were immediately capped tightly after addition of the methoxyflurane-containing buffer and placed in a shaking Dubnoff incubator at 37°C. Reactions were terminated at 30 minutes by immersing the vials in a boiling-water bath for 5 min. To insure that concentrations of methoxyflurane remained constant in the vials, occasional samples of the incubation mixtures were injected online into a Varian gas chromatograph equipped with a flame ionization detector.

After centrifugation and filtration of the incubation mixtures, inorganic free fluoride ion concentration was read with an Orion fluoride electrode and Corning pH meter. Five-minute equilibration of the electrode was allowed with each sample before the reading was determined. Hydrolysis was employed to determine organic fluoride-containing metabolites of methoxyflurane. Concentrated H2SO4 was added to the filtrates to lower the pH to 1.5. Filtrates were then incubated at room

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**Fig. 1.** Biotransformation of methoxyflurane to free fluoride and total fluoride metabolites as a function of concentration of anesthetic in non-induced microsomes. n = 32 for each point, ± SE.

**Fig. 2.** Biotransformation of methoxyflurane to free fluoride and total fluoride metabolites as a function of concentration of anesthetic in phenobarbital-pretreated microsomes. n = 32 for each point, ± SE.
Fischer 344 and Sprague-Dawley rats, data from the two groups were combined for final kinetic analysis.

The combined-species curves for non-induced biotransformation are shown in figure 1; the induced curves, in figure 2. Computer analysis demonstrates that two curves fit the observed results of methoxyflurane biotransformation equally well. One curve (upper panel, fig. 3) is an exponential asymptotic one with the empiric formula, \( y = \frac{x}{ax + b} \). The other curve (lower panel, fig. 3) is an unbounded exponential one with the empiric formula, \( y = a \ln(bx + 1) \). Both curves pass through zero and never show an absolute decrease in either free or total fluoride production even at the highest concentrations of methoxyflurane. (The 2.5 mM methoxyflurane concentration is near saturation in the buffer solution employed.) It could not be conclusively demonstrated which of these two model curves is applicable to the metabolism of the anesthetic, but certainly no substrate inhibition occurs. Either curve, however, clearly demonstrates that the rate of methoxyflurane biotransformation increases as higher concentrations are obtained, although the fraction biotransformed decreases with increasing concentrations.

When the ratios of total/free fluoride in the induced and noninduced preparations are compared at each anesthetic concentration, they differ at the \( P < 0.01 \) level (table 1). These data indicate that phenobarbital pre-treatment increases the proportion of free fluoride produced compared with the production of total (and hence organic) fluoride. Thus, phenobarbital induction not only increases biotransformation in a quantitative manner, it also enhances the free fluoride pathway of methoxyflurane biotransformation to a greater extent than it does the pathway to organic fluorine-containing metabolites.

Lineweaver-Burke double reciprocal plots were derived from the combined data of both total and free fluoride production for both induced and non-induced microsomal preparations (fig. 4). \( V_{\text{max}} \) and \( K_{\text{m}} \) of total and free fluoride were calculated by linear regression analysis (table 2). Phenobarbital increases total fluoride production from methoxyflurane 6.9-fold and increases free fluoride temperature for 18 hours, NaOH was then used to back-titrated to pH 7.0, and fluoride electrode readings were taken. This procedure measured total fluoride as the sum of free inorganic fluoride and organic fluoride.

All data were subjected to statistical analysis, including Student's t-test, linear regression analysis, and analysis of variance. A control data computer was used to analyze and to derive empirical formulas for the curves.

Results

There was no difference in production of free fluoride or total (organic plus inorganic fluoride) fluoride at any methoxyflurane concentration when the Sprague-Dawley and Fischer 344 rats were compared by analysis of variance. There were, however, great increases in total and free fluoride production by both strains following phenobarbital induction, although there was no difference between the two strains. Difference between induced and non-induced total and free fluoride production was at the \( P < 0.001 \) level at every concentration except 0.06 \( \mu \)M methoxyflurane, which differed at the \( P < 0.01 \) level of significance. Because the biotransformation of methoxyflurane was the same in
10.3-fold. When the ratio (total fluoride $V_{\text{max}}$/free fluoride $V_{\text{max}}$) is calculated, it is 3.45 for non-induced microsomes and 2.3 for induced microsomes. This demonstrates a proportionately greater production of inorganic fluoride than organic fluoride with phenobarbital induction.

**Discussion**

Van Dyke and Wood\(^\text{a}\) demonstrated that there are at least two distinct pathways of methoxyflurane biotransformation. In one pathway, inorganic fluoride release and ether scission constitute initial steps, whereas the other pathway results in organic fluorine-containing metabolites such as dihydroxymethoxyacetic acid, thought to be excreted in the urine without significant hepatic microsomal dehalogenation. With concentrations of methoxyflurane as high as 2.2 mM, these investigators were unable to detect substrate inhibition. Although they did not subject their concentration–product curves to analysis, there was graphic indication that these were exponential in form, roughly indicating first-order kinetics. The present study agrees that there are two distinct pathways of methoxyflurane biotransformation functioning through the microsomal mixed-function oxidase system, although it is not certain that two separate enzymes are involved. In fact, there is ample evidence that cytochrome P-450 alone is capable of a wide variety of oxidative degradations, including ether cleavage, alkane oxidation, and dehalogenations.\(^\text{a}\) This study, in concert with that of Van Dyke and Wood, confirms that there is no difference between the rates of hepatic microsomal biotransformation of methoxyflurane *in vitro* by Sprague-Dawley and Fischer 344 rats. The greater susceptibility of Fischer 344 rats to methoxyflurane nephrotoxicity observed by Mazze *et al.*\(^\text{a}\) is not the result of greater inorganic fluoride production by hepatic microsomal enzymes in this strain.

Many drugs are known to interact with the mixed-function oxidase system to inhibit the biotransformation of other drugs administered simultaneously. With most nonvolatile drugs (hexobarbital, ethylmorphine, chlorproma-

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<th>Table 1. Ratios, Total Fluoride/Free Fluoride*</th>
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<td>Methoxyflurane Concentration (mM)</td>
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* Ratios of total fluoride/free fluoride at various methoxyflurane concentrations. Each ratio is the mean from eight preparations. The mean of the induced and pretreatment with phenobarbital ratios differs at the $P < 0.01$ level of significance.

![Fig. 4. Lineweaver-Burke plots of methoxyflurane biotransformation. A, free F⁻, non-induced microsomes; B, total F⁻, non-induced; C, free F⁻, phenobarbital-pretreated; D, total F⁻, phenobarbital-pretreated. X-axis is mMolF⁻/mg microsomal protein/30, while y-axis is mM methoxyflurane.](image-url)
zinc, xoxazolamine, phenylbutazone, etc.), one drug inhibits the metabolism of the other in a competitive manner. Kinetics of single "fixed" drugs indicate metabolism follows exponential first-order kinetics. Inhalation anesthetics such as halothane inhibit the biotransformation of fixed drugs such as barbiturates in vitro. However, the inhibition of the mixed-function oxidase system by inhalation anesthetics is noncompetitive, implying an action over and above simple competition for enzymatic active sites. True first-order kinetics for methoxyflurane can be suspected but not confirmed on the basis of the present study. It could be speculated that lipid-soluble inhalation anesthetics change the molecular configuration of phospholipid membranes contiguous to microsomal enzyme active sites, thus producing a type of allosteric inhibition. However, this thesis has yet to be conclusively documented.

The inhibitory site of inhalation anesthetics on the hepatic microsomal electron-transport chain is at cytochrome P-450, since neither methoxyflurane nor halothane nor enflurane decreases the in vitro linear activity of NADPH cytochrome c reductase or microsomal cytochrome b5 even at saturating concentrations. This study confirms that there is no substrate inhibition by methoxyflurane, when metabolite formation is measured. At higher concentrations of the anesthetic the rate of increase of biotransformation slows, but biotransformation continues to increase. These findings are in conflict with those of Sawyer et al. This group used an in-vitro preparation that contains inherent variables not present in vitro. In addition, they did not look at metabolite formation as an end point, but rather at the fraction of anesthetic sequestered in the liver.

The data from these experiments do not distinguish whether biotransformation at high methoxyflurane concentrations continues in a linear fashion in the secondary slope (unbounded curve) or whether it approaches an asymptote. If applied to the in-vivo situation this is an academic point, since the higher concentrations of methoxyflurane are near saturation.

An unexpected result of this study was demonstration of a shift in the pathway of methoxyflurane metabolism produced by phenobarbital induction. Phenobarbital pre-treatment enhances the pathway to inorganic free fluoride metabolite production to a greater extent than it does the pathway to organic fluorine-containing products. It has generally been assumed with all drugs that microsomal enzyme induction produces a purely quantitative change in biotransformation. This study is believed to be the first to show that methoxyflurane biotransformation not only is quantitatively increased by induction, but also is qualitatively altered by enhancement of the pathway of inorganic fluoride-containing metabolites, as determined by direct measurement.

References


### Table 2. \( V_{\text{max}} \) and \( K_m \) Values, Non-induced and Induced Microsomal Metabolism of Methoxyflurane*

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<td></td>
<td>( V_{\text{max}} )</td>
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<td>135.1</td>
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* \( V_{\text{max}} \) values are in mmol F-/mg microsomal protein/30; \( K_m \) in mM; \( n = 8 \) for each value.
METHOXYFLURANE BIOTRANSFORMATION


Transfusion

MICROPOROUS FILTERS Two groups of dogs (seven animals each) underwent exchange transfusions of twice the calculated blood volume in each dog. Dacron wool (swank) filters were used in one group (DW) and polyester mesh (Pall) filters were used in the other group (PM). Bank blood stored at 4°C for five days was used. Screen filtration pressure (SFP) of transfused blood was elevated, 424 and 370 mm Hg in groups DW and PM, respectively. There was a significant decrease in pH following transfusion in group PM compared with pretransfusion values. Oxygen consumption and QO2 did not change significantly in either group following transfusion, but post-transfusion O2 consumption was significantly lower and QO2 significantly higher in group PM compared with group DW. There were significant increases in pyruvate and lactate concentrations following transfusion in group PM but not in group DW. Pulmonary diffusing capacity for oxygen was significantly lower following transfusion in group PM. After filtration, average SFP’s were reduced to 4 and 26 mm Hg in groups DW and PM, respectively. There were significant reductions in platelet counts following transfusion in both groups. A single isolated embolus was found in the lung of one animal in group DW, while six of seven dogs in group PM had pulmonary emboli. It was concluded that although greater volumes of blood could pass through the Pall filter compared with the Swank filter, this was at the expense of permitting administration of small microaggregates, and that these could be completely prevented by the use of dacron wool blood transfusion filters. (Barrett J, and others: A comparison in vivo of dacron wool (Swank) and polyester mesh (Pall) microporous blood transfusion filters in the prevention of pulmonary microembolism associated with massive transfusion. Ann Surg 182:690–695, 1975.)