Metabolism of Methoxyflurane: 
Release of Inorganic Fluoride in Human and Rat Hepatic Microsomes

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The amount of inorganic fluoride released through the enzymatic metabolism of methoxyflurane was examined in vitro in microsomes obtained from rat and human livers. The human microsomal enzyme system showed a high degree of individual variation which did not correlate with any observable factor, suggesting a dissimilarity in the microsomal enzymes among individuals. The studies in rats showed a high uniformity in enzymatic activity for the release of inorganic fluoride from methoxyflurane. Furthermore, this uniformity was evident when three strains were compared (Long-Evans, Fischer 344, and Sprague-Dawley), suggesting a similarity in the microsomal enzymes that release inorganic fluoride in these strains. The results in human and rat microsomes indicate that there is no substrate inhibition for the release of inorganic fluoride. Incubation of microsomes with methoxyflurane resulted also in the production of material that was degraded in an acidic medium to produce both formaldehyde and additional inorganic fluoride. The data suggest that modification of our concept of the pathway of methoxyflurane metabolism is necessary. (Key words: Methoxyflurane; Biotransformation; Human microsomes in vitro; Fluoride; Formaldehyde.)

The biotransformation of methoxyflurane has assumed new importance since the reports by Taves et al.1 and more recently Mazze and Cousins2 that inorganic fluoride, one of the products, is responsible for a polyuric renal dysfunction sometimes seen after clinical use of this anesthetic. However, many questions regarding the metabolism of this agent and, in particular, the manner in which the enzymatic attack takes place, remain unanswered.

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It has been suggested by Halsey and co-workers,3 on the basis of studies of halothane by Sawyer et al.,4 that the metabolism of a number of the volatile anesthetics is decreased as the concentration of the anesthetic in the blood increases, an indication that the substrate inhibits the enzyme at high concentrations. This conflicts with the report by Taves et al.,1 who suggested a dose-related nephrotoxicity for methoxyflurane, which, if caused by inorganic fluoride production from the anesthetic, would indicate a linear relationship between fluoride production and methoxyflurane concentration and argue against substrate inhibition at the enzyme level.

There also is some question concerning the number of enzymes necessary for the complete degradation of the methoxyflurane molecule. The enzyme system for the dechlorination of the molecule has been described,5 but it is not known whether the ether cleavage and the associated release of inorganic fluoride are catalyzed by the same enzyme system; some evidence which indicates that there are two enzymes has been reported.6 If multiple enzyme systems are necessary for the complete degradation of methoxyflurane, there is an increased possibility of species and strain differences due to differences in relative activities of the enzymes.

The usual method used to follow the biotransformation of methoxyflurane has been to quantitate the blood and urine levels of inorganic fluoride. Mazze et al.7 found that blood and urine levels of fluoride are similar in different strains of rats even though these strains vary in their susceptibility to polyuric renal dysfunction. Because the blood and urine levels are the net result of the production of inorganic fluoride and its uptake by tissues other than those in which it is produced, measuring the appearance of inorganic fluoride in blood and urine may not reflect its rate of production at the enzyme level. This
can only be measured in vitro with subcellular fractions.

Therefore, we used the microsomal enzyme system known to be the active metabolizing system for a number of drugs. This system has previously been found to be active in the dechlorination and ether cleavage of the volatile anesthetics.  

**Methods**

**ANIMAL TISSUE**

Adult male rats of the Sprague-Dawley, Fischer 344, and Long-Evans strains were used. Those treated with phenobarbital were given intraperitoneal injections, 50 mg/kg, once a day for 2 days and were sacrificed 24 to 48 hours after the last treatment. Rats treated with methylcholanthrene were given a single intraperitoneal injection, 20 mg/kg in corn oil, 24 hours before they were sacrificed.

After decapitation of the rats, the livers were quickly removed and homogenized at 0 to 5°C with 3 vol of 0.15 per cent KCl. The homogenate was centrifuged for 15 minutes at 9,000 × g, and the supernatant fraction was decanted and centrifuged at 105,000 × g for 1 hour, isolating the microsomes as a pellet. The microsomal pellet was resuspended in 0.05 M tris buffer, pH 7.4. The supernate of the 105,000 × g centrifugation contains all the soluble materials of the cell.

**HUMAN LIVER**

Human liver samples were obtained at necropsy, within 5 hours of death. Human liver samples also were obtained from cadaveric kidney donors after the removal of the kidneys. The liver samples were immediately cooled and the microsomes were prepared according to the procedure outlined above for the rat livers.

**INCUBATION CONDITIONS**

Equal portions of the microsomal suspension were measured into polyethylene vials,
ethylene diamine tetraacetate was added to a final concentration of 10^{-9} M, and the vials were flushed with oxygen. Methoxyflurane was added to the samples as a measured amount of methoxyflurane-saturated tris buffer (pH 7.4). The total volume in each incubation vial was adjusted to 2.5 ml by addition of tris buffer. The final microsomal protein concentration, measured by the method of Lowry et al., was between 4 and 8 mg/ml.

In some incubations the flasks were flushed with 80% nitrous oxide-20% oxygen for 30 seconds. In other incubations, tetracycline was added to a final concentration of 0.9 mm.

After the sample had been warmed to 37 C, the reaction was started by addition of an NADPH-generating system consisting of 2 μmol of NADP, 5 μmol of glucose-6-phosphate, and 2.5 μl (0.75 unit) of glucose-6-phosphate dehydrogenase per sample. The closed reaction vessels were incubated, with mixing, for 15 minutes.

At the end of the incubation, a 1.0-ml amount was taken for determination of the methoxyflurane concentration by extraction. With hexane and gas chromatography. A Barber-Coleman gas chromatograph was used with a 1.7-m column of 100 to 120 mesh Carbowax 400/Porasil C (Waters) at 103 C. An electron capture detector was used, and argon-methane was the carrier gas.

The remaining incubation mixture was cooled to room temperature, and the fluoride content was measured with a fluoride-specific electrode (Orion models 94-09 fluoride electrode and 90-02 reference electrode) with a Beckman SS-2 pH meter. An equilibration time of 5 or 10 minutes was allowed; after each sample measurement, the electrodes were rinsed with distilled water until the potential was greater than 180 mV. Microsomal suspensions of known fluoride concentrations were prepared from 0.1 M fluoride standard solution obtained from Orion and used for daily calibration curves. Concentrations in the micromolar range could be accurately determined.

Acid-labile metabolites of methoxyflurane were broken down by the addition of 10 μl of concentrated sulfuric acid to 1.5 ml of incubation mixture, which brought the pH to about 1.5. On the following day the pH was re-adjusted to 7.0 with NaOH, resulting in a volume no greater than 1.8 ml. Fluoride measurements were made according to the previously described procedure. These samples were also assayed for formaldehyde according to the method described by Nash.

**Fig. 3. Effect of pretreatment of rats with enzyme-inducing agents. Data are shown as mean ± SE. The phenobarbital value is significantly different from control, P < 0.02.**

**Results**

There was no obvious pattern to the enzymatic activity that produced inorganic fluoride from methoxyflurane in vitro in human microsomes (fig. 1). Neither age nor sex influenced the results and, more important perhaps, the enzymatic activity was not decreased with increasing substrate concentrations, thus supporting the findings of Taves et al., and Mazze and Cousins that increasing meth-

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1 Inorganic fluoride release was proportional to the amount of microsomal protein up to a protein content of 8 mg/ml; above this, the relationship was not linear, and therefore all incubations were carried out with microsomal protein concentrations of 4 to 8 mg/ml.
oxyflurane concentrations led to higher fluoride levels. However, figure 1 also shows the large variation in enzymatic activity among individuals.

Figure 2 compares the fluoride-releasing activity of microsomes from the Fischer 344 and Sprague-Dawley strains of rats. There was no detectable difference between the rates of release of fluoride. The Long-Evans strain showed the same enzymatic activity. As with the human microsomes, there was no substrate inhibition. Individual variation was small compared with the human data.

The effects of the enzyme-inducing agents, phenobarbital and methylcholanthrene, on fluoride production in rats are presented in figure 3. All three strains showed a uniform induction pattern with phenobarbital. Methylcholanthrene treatment does not induce all the enzyme systems induced by phenobarbital, and therefore the lack of inducing effect in this instance is not surprising.

The amounts of formaldehyde produced in these incubations proved to be at the lower end of the sensitivity of the formaldehyde assay and therefore the results are not reliable.

Both nitrous oxide and tetracycline inhibited fluoride release (table 1). Previous studies have shown that the dechlorinating enzyme system is stimulated considerably by a factor in the soluble portion (105,000 × g supernate; see Methods) of the cell. Therefore, to determine whether the enzymes which release the inorganic fluoride are similar to those which dechlorinate, we examined the effect of the presence of the soluble portion of the cell on the inorganic fluoride production. We found it to have no significant effect (table 1).

In addition to inorganic fluoride, the metabolism of methoxyflurane yields a metabolite which, when treated with acid, breaks down to release additional inorganic fluoride and formaldehyde. Some formaldehyde is produced as a result of enzymatic activity, but most of this is quickly oxidized to CO₂, thus explaining the low levels of formaldehyde produced enzymatically. A comparison of the enzymatically produced inorganic fluoride and formaldehyde with the inorganic fluoride and formaldehyde released by acid is after incubation, 3.5 μmol of fluoride and approximately 0.75 μmol of formaldehyde; acid treatment after incubation, 8.2 μmol of fluoride and 4.1 μmol of formaldehyde.

Figure 4 shows the pH optimum for the release of fluoride during incubation in vitro to be 8.0, good evidence that this is an enzymatic release and is separate from the acid-labile fluoride.

**Discussion**

One of the more puzzling aspects of the nephrotoxicity that occurs after use of methoxyflurane has been the variation in its incidence. In previous reports, this variation has been considered to be dose-related. Although no doubt important, this does not take into account the individual differences in enzymatic activity which we report here. It is evident that the amount of fluoride produced is related to the amount of methoxyflurane present, but some individuals have more active enzyme systems than others and, thus, could attain high serum fluoride levels more readily. There seemed to be no apparent correlation of enzymatic activity with sex or age.

Mazze *et al.* found that rats of different strains respond differently to the nephrotoxic effects of inorganic fluoride. In their studies, the Fischer 344 strain was susceptible to polyuria at fluoride levels which did not affect the Sprague-Dawley or Long-Evans strains. Likewise, the Fischer 344 rats were susceptible to polyuria caused by fluoride production from the metabolism of methoxyflurane, while other strains were not, even though similar serum and urine fluoride levels were attained in all strains. We have found that there is no difference in the enzymatic activities responsible for releasing the fluoride from methoxyflurane in these three rat strains. Some
studies\textsuperscript{10} have shown that, in the case of certain drug oxidations, although enzymatic activities are apparently the same in normal animals of different strains, they are not always the same after enzyme induction. However, the enzymatic activities in these three rat strains responded equally to enzyme induction with phenobarbital.

The strain differences in the development of the polyuria probably represent a more complex situation than just the production of high levels of inorganic fluoride. These differences may represent a variation in the sensitivity of the kidneys to fluoride, probably due to a difference in physiologic factors. In support of the fact that conditions within the kidney play a role in the sensitivity of the kidney to inorganic fluoride are the studies with tetracycline. It has been reported that patients given tetracyclines before or after methoxyflurane anesthesia have an extremely high incidence of polyuria.\textsuperscript{11} If the inorganic fluoride were the only causative factor in polyuria, one would suspect that the tetracycline would enhance the defluorination of methoxyflurane. This report shows this not to be the case. Therefore, it may be that tetracycline or one of its metabolites sensitizes the kidneys to the fluoride produced from methoxyflurane.

One point which should be mentioned is that the tetracyclines are dispensed with large amounts of ascorbic acid for stabilization, thereby adding an extra unknown element in that little is known about the effect of ascorbic acid on the fluoride-kidney effect.

Previous studies of the pathway of methoxyflurane metabolism have indicated the following products: inorganic fluoride,\textsuperscript{1} carbon dioxide,\textsuperscript{12} dichloroacetic acid,\textsuperscript{12} methoxydifluoroacetic acid,\textsuperscript{12} oxalic acid.\textsuperscript{14} Because of their highly polar nature, it is likely that neither dichloroacetic acid nor methoxydifluoroacetic acid is further oxidized enzymatically, leading to the conclusion that oxalic acid is not the product of an enzymatically controlled reaction but may arise nonenzymatically. For this reason, we offer a revised scheme for the metabolism of methoxyflurane which takes into account recent findings:

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\begin{align*}
? & \xrightarrow{Enzyme \text{ A}} [\text{CH}_3\text{O-CF=CCl}_2] + F^- \\
\text{CH}_3\text{O-CF}_2\text{CCl}_2\text{H} & \xrightarrow{Enzyme \text{ B}} \text{CH}_3\text{O-CF}_2\text{COOH} + 2\text{Cl}^- \\
\text{HCHO} + 2F^- + \text{HCl}_2\text{COOH} & \rightarrow \text{CO}_2 + \text{Urinary product} \\
\text{HCHO} + 2F^- + \text{HOOC-COOH} & \rightarrow \text{Urinary product}
\end{align*}
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However, some thought should be given to how the oxalic acid arises.

Fry et al. have reported that a urinary metabolite of methoxyfluorane produces inorganic fluoride when treated with acid. The present studies support these findings and indicate that the enzymatic metabolism of methoxyflurane in vitro yields a compound which, when treated with acid, produces not only inorganic fluoride but also formaldehyde, thus indicating that the acid-labile compound is a methyl ether.

At least two possibilities exist as to the nature of the acid-labile compound—methoxydifluoroacetic acid (CH$_3$—O—CF— COOH) and methoxyfluorodichloroethylene (CH$_3$—O—CF=CCl$_2$), both of which are considered to be acid-labile (E. R. Larson, personal communication), and both of which would yield inorganic fluoride and formaldehyde. Unfortunately, neither compound is available for further study. Obviously, either of these compounds could also be a source of oxalic acid, from the ethyl or ethylene portion of the ether, as a result of the breakdown of this compound in acid. We are continuing these studies to determine more precisely the nature of the acid-labile material.

The present study clearly shows that the metabolism of methoxyflurane is subject to large individual variations but is not inhibited by high substrate concentrations, and also supports the fact that there are only minor strain differences among rats in the enzymatic activity that releases fluoride from methoxyflurane. The present study also supports the production of an acid-labile metabolite. These data are used to revise the pathway of metabolism of methoxyflurane.

References


