Nonspecific Stimulation of Drug Metabolism in Rats by Methoxyflurane

M. Lawrence Berman, M.D., Ph.D.,* and Julius F. Bochantin, B.S.

Pretreatment of rats with a subanesthetic concentration (0.13 per cent) of methoxyflurane vapor, seven hours per day, resulted in the following responses relative to a control group: 1) hexobarbital sleeping time was reduced by 79 per cent when rats were exposed to the subanesthetic concentration for 15 days; 2) aminopyrine demethylase activity was markedly increased in the 9,000 × g supernate fraction of liver homogenates prepared from rats after they had been exposed for ten days; 3) a significant number of rats pretreated for ten days subsequently survived a lethal concentration of methoxyflurane, based on determination of the LD₅₀. These effects of methoxyflurane were reversed by an inhibitor of microsomal enzymic activity. These results suggest that methoxyflurane is a nonspecific stimulator of drug-metabolizing enzymes. In the 9,000 × g supernate, which contained only the microsomes and soluble cellular material, there was a marked increase in protein content, accompanied by a significant rise in drug-metabolizing activity. The increased protein may reflect an increase in microsomal enzymes and, therefore, enzyme induction. (Key words: Methoxyflurane; Hexobarbital sleeping time; Aminopyrine demethylase; Liver microsomes; Metabolism; Enzyme induction.)

Many drugs that induce their own metabolic breakdown by hepatic microsomes also stimulate the metabolic destruction of other pharmacologic agents.¹ ² Methoxyflurane induces its own metabolism,⁴ but its ability to enhance the metabolism of other drugs by hepatic microsomes has not been demonstrated. This investigation was designed to determine if methoxyflurane is a nonspecific stimulator of hepatic microsomal enzymes. We found evidence that chronic exposure of rats to a subanesthetic concentration of methoxyflurane vapor shortens the hypnotic effect of hexobarbital, decreases the mortality of rats exposed to a lethal dose of the anesthetic, and stimulates oxidative demethylation by a 9,000 × g supernate fraction of liver homogenates.

Methods

Male Sprague-Dawley rats which initially weighed 40 to 50 g were used throughout this study. The animals were maintained on a stock diet and housed in metal cages in an air-conditioned room maintained at constant temperature. Care was taken to prevent exposure of the animals to materials known to affect drug-metabolizing enzymes, such as chlorinated insecticides ⁴ and cedar wood shavings.⁵

Exposure Chamber. The chamber used for administering methoxyflurane vapor to rats was a 55-l rectangular glass-sided aquarium with an inlet and an outlet at diagonally opposite ends. The aquarium could accommodate as many as 30 rats at once. Methoxyflurane in 10 l/min of filtered air was delivered through the inlet from a Pentec vaporizer. To obtain a subanesthetic concentration of methoxyflurane vapor that did not interfere with the growth of the rats, the dial of the Pentec was turned to a point just in advance of the "off" position. At this setting, the concentration of methoxyflurane in representative gas samples obtained from the aquarium was 0.13 per cent (±0.04 per cent) as determined by chromatographic analysis. The concentration was constant at 1, 3.5, and 6.5 hours during seven hours of exposure.

The control rats were placed in an identical, but separate, aquarium. They breathed only filtered air at 10 l/min.

Concentrations of carbon dioxide in the chambers, determined by gas chromatography, were 0.3 per cent after the rats had been exposed to 0.13 per cent concentration of the anesthetic for seven hours, and 0.35 to 0.4 per cent after the control rats had been exposed to air for a similar period.

* Assistant Professor.
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Three experiments on the influence of pretreatment with methoxyflurane were performed, to determine: 1) duration of hexobarbital sleeping time; 2) tolerance to a lethal concentration of methoxyflurane; and 3) activity of aminopyrine demethylase in the 9,000 × g supernate from rat liver homogenates. In addition, the effect of butylated hydroxytoluene (BHT) on hexobarbital sleeping time was determined. BHT is the preservative in commercial methoxyflurane.

**Hexobarbital Sleeping Time**

This was used as a screening test to determine if commercial methoxyflurane affected drug-metabolizing enzymes. One group of rats was exposed to 0.13 per cent methoxyflurane vapor for seven hours per day and a control group was exposed to air for seven hours per day. At the end of the seven hours the rats were returned to their cages and permitted to eat and drink ad libitum. Five or six rats from each group were used to measure the duration of action of hexobarbital 24 hours after the above treatment. This test was repeated after 1, 2, 3, 4, 5, 10 and 15 consecutive days of exposure to methoxyflurane. Each rat was given 125 mg/kg of sodium hexobarbital in physiologic saline solution intraperitoneally (ip) and the time interval until return of the righting reflex was measured. A final group of rats was exposed to methoxyflurane as described for ten consecutive days. Twenty-four hours after their last exposure and 40 minutes prior to determining the hexobarbital sleeping time each rat was given an ip injection of 50 mg/kg SKF 525-A * (beta-diethylamino phenylpropylacetate HCl) dissolved in physiologic saline solution. Rats employed in estimating the duration of action of hexobarbital were used only once and then destroyed.

**Tolerance to a Lethal Concentration of Methoxyflurane**

Rats, in groups of six, were pretreated with 0.13 per cent methoxyflurane seven hours per day for ten consecutive days. Ninety-six hours later, these rats were exposed to a predetermined lethal dose of methoxyflurane (0.5 per cent). Another group of rats was pretreated with 0.13 per cent methoxyflurane as described. Forty minutes prior to exposure to the lethal concentration of the anesthetic, each rat was given an ip injection of SKF 525-A, 50 mg/kg.

The lethal dose of methoxyflurane was based on an LD_{50} determined by exposing rats in groups of six to methoxyflurane in air at 10 l/min. An unexposed group of rats was used for each of several concentrations of the anesthetic: 0.13, 0.21, 0.27, 0.38 and 0.50 per cent. The number of dead animals at the end of five hours was counted. This experiment was repeated five times for each concentration (table 1) at comparable times on different days, and the sequence of exposure was randomized. Probit analysis was used to calculate the LD_{50}. This method of determining the LD_{50} of methoxyflurane vapor was repeated using rats pretreated with an ip injection of SKF 525-A, 50 mg/kg, 40 minutes prior to exposure to a given concentration of methoxyflurane.

**Activity of Aminopyrine Demethylase in the 9,000 × g Supernate**

Rats were divided into three groups of 12 each. One group was exposed to 0.13 per cent methoxyflurane seven hours per day for ten consecutive days; 24 hours after the last exposure these rats were killed by decapitation. The second group was treated similarly except that 40 minutes prior to decapitation each

### Table 1. Results of Exposure to Various Concentrations of Methoxyflurane for Five Hours

<table>
<thead>
<tr>
<th>Methoxyflurane Concentration (Per Cent)</th>
<th>Per Cent Mortality* Without SKF 525-A</th>
<th>Per Cent Mortality* With SKF 525-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.13</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.21</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>0.27</td>
<td>33</td>
<td>27</td>
</tr>
<tr>
<td>0.38</td>
<td>86</td>
<td>50</td>
</tr>
<tr>
<td>0.50</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

LD_{50} (Per Cent) 0.31 (Probit analysis)

95 (Per Cent) confidence limits 0.40-0.29 0.35-0.24

* 30 rats were used at each concentration.
rat was given 50 mg/kg of SKF 525-A ip. The third, or control, group was exposed to air for seven hours per day for ten consecutive days.

After thorough bleeding, the livers of all rats were excised, blotted on filter paper previously refrigerated (0 C) and moistened with ice-cold 0.25-M sucrose. The organs were then quickly cut into small pieces and placed in refrigerated previously-teared beakers containing ice-cold 0.25-M sucrose. The beakers were then weighed. The liver pieces with the sucrose solution were transferred to ice-cold glass homogenizer cups and additional ice-cold 0.25-M sucrose was added sufficient to prepare 20 per cent homogenates. This was done in a cold room (0 to 4 C) using a motor-driven teflon-coated pestle. Homogenates were centrifuged for 20 minutes at 9,000 x g at 0 C.

The ability of the 9,000 x g supernate to demethylate aminopyrine was determined by the amount of formaldehyde formed using the Nash reagent by the technique of Coehlin and Axelrod. The reaction mixture was prepared as described by Argyris. The incubation time was 90 minutes. All assays were done in triplicate. Formaldehyde standards were used with every determination. Protein was assayed in triplicate on the 9,000 x g supernate fraction, using the biuret reaction.

Butylated hydroxytoluene (BHT) increased the activity of hepatic microsomal enzymes which metabolize hexobarbital when BHT was given orally to rats in large doses for several days. To determine if BHT administered ip affected the hexobarbital sleeping time, 36 rats were divided into six groups; one group served as a control, and each rat in the other five groups received an ip injection of BHT dissolved in 0.25 ml of corn oil twice a day for five days. On the sixth day hexobarbital sleeping time was determined. The dosage schedule for BHT was: Group 1, 0.001 mg; group 2, 0.01 mg; group 3, 0.1 mg; group 4, 1 mg; group 5, 10 mg; group 6, 0.25 ml corn oil only.

Results

Rats exposed to 0.13 per cent methoxyflurane vapor seven hours per day for as long as 15 consecutive days gained as much weight as control rats.

Hexobarbital Sleeping Time

Pretreatment with methoxyflurane markedly shortened the hypnotic action of hexobarbital (table 2). This effect appeared after two days of treatment and persisted over the entire period studied. The hexobarbital sleeping time decreased 79 per cent between the first and the fifteenth day of methoxyflurane treatment. SKF 525-A prolonged the duration of action of hexobarbital beyond that in the controls (table 3).

Tolerance to a Lethal Concentration of Methoxyflurane

The difference between the LD₅₀ of methoxyflurane in normal rats and that in normal rats pretreated with SKF 525-A was not significant (table 1). Exposure of both groups of rats to methoxyflurane vapor, 0.5 per cent,
TABLE 3. Mean Hexobarbital Sleeping Times of Rats Following Treatment with Methoxyflurane Vapor and SKF 525-A

<table>
<thead>
<tr>
<th>Number of Consecutive Days of Exposure (Seven Hours per Day)</th>
<th>Number of Rats</th>
<th>Control Sleeping Time ± SD (minutes)</th>
<th>Sleeping Time after Methoxyflurane 0.13 per cent. ± SD</th>
<th>Number of Rats</th>
<th>Sleeping Time after Methoxyflurane, 0.13 per cent; SKF 525-A, 50 mg/kg, ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>6</td>
<td>105 ± 14.5</td>
<td>22 ± 3.0</td>
<td>6</td>
<td>144 ± 11.5</td>
</tr>
</tbody>
</table>

resulted in 100 per cent mortality. However, mortality at this concentration was reduced 43 per cent if the rats were first exposed to a subanesthetic concentration of the anesthetic. The decrease in mortality of pretreated rats after 0.5 per cent methoxyflurane was abolished by SKF 525-A.

**Activity of Aminopyrine Demethylase in the 9,000 × g Supernate**

There was a marked increase in N-demethylase activity in the 9,000 × g supernate of liver homogenates obtained from rats which had been pretreated with a subanesthetic concentration of methoxyflurane, compared with the activity in the same fraction obtained from control rats (table 5). SKF 525-A significantly inhibited N-demethylase activity in the 9,000 × g supernate prepared from livers of rats pretreated with methoxyflurane. Enzymic activity in the 9,000 × g supernate from rats given the inhibitor was significantly lower than control values.

The amount of protein in the hepatic microsomes from rats pretreated with methoxyflurane was markedly increased (table 6). However, livers of the drug-treated rats were not significantly heavier than livers from controls. The mean wet weights of the control and drug-treated rat livers were 5.5 ± S.D. 0.56 g and 5.3 ± S.D. 0.31 g, respectively. SKF 525-A as used in these experiments did not affect the amount of 9,000 × g supernatant protein (mean weight 767.5 ± S.D. 68.24 mg), nor did it affect liver growth (mean wet weight 5.0 ± S.D. 0.48 g).

BHT administered to rats in a dose of 10 mg twice a day for five days resulted in a marked reduction in the hexobarbital sleeping time, 28.8 ± S.D. 8.1 min, compared with the control value, 89.8 ± S.D. 23.8 min. The other doses employed failed to affect hexobarbital sleeping time.

**Discussion**

Van Dyke demonstrated that chronic exposure of rats to a subanesthetic concentration of methoxyflurane vapor enhanced the ability of hepatic microsomes to dechlorinate methoxyflurane. The work described in this paper indicates that methoxyflurane is also a nonspecific stimulator of drug metabolism. The observation that the hexobarbital sleeping time was significantly decreased in rats pretreated with methoxyflurane is indirect evidence for this conclusion. The hepatic microsomal enzyme inhibitor SKF 525-A reversed this effect.

The use of the hexobarbital sleeping time as a screening test to determine if methoxyflurane affected microsomal enzyme systems other than the dechlorinating system was based on the report that the duration of hexobarbital hypnosis is largely regulated by the level of hepatic microsomes that inactivate this drug. Many drugs known to stimulate drug-metabolizing enzymes by hepatic microsomes also stimulate metabolism of hexobarbital. SKF 525-A prolongs the duration of action of hexobarbital by inhibiting hepatic microsomal enzymatic activity, without producing sedative effects of its own or eliciting any other physiologic response in the dose used.

Direct evidence for the nonspecific stimulating effect of methoxyflurane on drug metabolism was the marked increase in aminopyrine demethylase activity in the 9,000 × g supernate prepared from livers of rats pretreated with a subanesthetic concentration of the anesthetic. Aminopyrine demethylase activity has been demonstrated in rat hepatic microsomes (9,000 × g fraction). Pretreatment of rats with many pharmacologic agents enhances the activity of this enzyme. Recent evidence suggests that aminopyrine demethylase activity may be inhibited by excess aminopyrine in the incubation medium. La
Table 4. Effect of Pretreatment on Mortality after Five Hours of 0.5 Per Cent Methoxyflurane

<table>
<thead>
<tr>
<th>Number of Rats</th>
<th>Pretreatment</th>
<th>Mortality (Per Cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>21</td>
<td>0.13 per cent methoxyflurane, 7 hours/day × 10 days</td>
<td>43</td>
</tr>
<tr>
<td>12</td>
<td>0.13 per cent methoxyflurane, 7 hours/day × 10 days; SKF 525-A, 50 mg/kg</td>
<td>100</td>
</tr>
</tbody>
</table>

Du et al. found that demethylation of aminopyrine by hepatic microsomes occurs in two steps: 1) demethylation of aminopyrine to monomethyl antipyrine (MMAP) and formaldehyde; 2) demethylation of MMAP to 4-aminantipyrine (4-AAP) and formaldehyde. Step 2 was found to be inhibited by $2 \times 10^{-3}$ M aminopyrine, but maximum formaldehyde formation required more than this concentration of substrate. Gram et al. noted that the amount of 4-AAP formed, compared with the amount of formaldehyde formed, was 3 per cent after 30, and 12.5 per cent after 90, minutes of incubation of $2 \times 10^{-3}$ M aminopyrine with rat hepatic microsomes (9,000 g fraction). Since $5 \times 10^{-2}$ M aminopyrine was used in our study, the amount of 4-AAP formed was probably much less than 12.5 per cent, because $5 \times 10^{-3}$ M aminopyrine inhibits the formation of 4-AAP. Therefore, formaldehyde formation presumably resulted from reaction 1, with an insignificant amount of 4-AAP formed during the incubation period. Since we found a significant increase in formaldehyde formation in the 9,000 g supernate of livers prepared from rats pretreated with methoxyflurane, compared with untreated controls, quantitative estimation of formaldehyde as an index of aminopyrine demethylase activity under these conditions seems justified.

Administration of SKF 525-A to rats inhibits aminopyrine demethylase activity. Aminopyrine demethylase activity was inhibited in rats pretreated with methoxyflurane and given SKF 525-A. Only pretreated rats were given SKF 525-A, as in the experiment to determine the hexobarbital sleeping time. This was done because it had been reported that SKF 525-A sometimes fails to inhibit enzymatic activity in microsomes prepared from control rats, but is an excellent inhibitor of enzymatic activity in microsomes prepared from drug-treated rats. In this study SKF 525-A was used to confirm that the effects of pretreatment with methoxyflurane were related to microsomal enzymatic activity.

The 9,000 g supernatant fraction of a hepatic homogenate contains only microsomes and soluble cellular material. In this fraction prepared from livers of rats pretreated with methoxyflurane there was a marked increase in protein content, accompanied by a significant rise in drug-metabolizing activity. The increased protein may reflect an increase in microsomal enzyme and, therefore, induction. Evidence that drug-induced increases in microsomal enzymatic activity represent increases in enzymic protein has been reported. However, from our data it is not possible to conclude unequivocally that methoxyflurane induced microsomal enzymes.

There was no significant increase in hepatic weight in rats pretreated with methoxyflurane. This finding is inconsistent with reports that many inducers of drug metabolism by hepatic microsomes also cause increases in hepatic weight. In one study chronic administration of an anesthetic concentration of methoxyflurane to mice increased hepatic weight but failed to stimulate drug-metabo-

Table 5. N-Demethylase Activity in Livers from Rats Exposed to Methoxyflurane

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>µg of Formaldehyde Formed per g Wet Weight of Liver (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>1.16 ± 0.86</td>
</tr>
<tr>
<td>Methoxyflurane</td>
<td>0.13 per cent 7 hours/day × 10 days</td>
<td>3.65 ± 1.20</td>
</tr>
<tr>
<td>Methoxyflurane: SKF 525-A</td>
<td>0.13 per cent 7 hours/day × 10 days</td>
<td>0.78 ± 0.50</td>
</tr>
</tbody>
</table>

* Control and methoxyflurane, $P < .001$.

Methoxyflurane and methoxyflurane-SKF 525A, $P < .001$.

Control and methoxyflurane-SKF 525A, $P < .025$.

† Six assays per treatment. Each assay represents the livers from three rats.
lizing enzymes in the liver, as determined by activity of a TPNH oxidase system. However, the discrepancies between the results of that study and those of our study may be explained by differences in the species used, concentrations of methoxyflurane employed, frequency and duration of exposure to anesthetic, and/or methods employed to detect drug-metabolizing activity.

The similarity between the values of LD$_{50}$ of methoxyflurane in normal rats with and without pretreatment with SKF 525-A indicated that this agent exerted no additive or synergistic effects on the dose-response curve of methoxyflurane. This finding is consistent with reports$^{12-15}$ demonstrating that SKF 525-A possesses no (or minimal) pharmacologic activity except that of an inhibitor of microsomal enzymic activity. Therefore, the observation that pretreatment of rats with a subanesthetic concentration of methoxyflurane vapor significantly reduced their mortality when the rats were exposed to a concentration of anesthetic lethal to control rats, and the blocking of this response by SKF 525-A, strongly suggest that the reduced mortality may be related (in part) to enhanced drug metabolism. The data in this study do not exclude the possibility that factors other than drug metabolism contributed to the increased survival after a lethal dose of methoxyflurane.

A minimum of 24 hours was necessary between the time the rats had been given their last pretreatment with a subanesthetic concentration of methoxyflurane and the determination of hexobarbital sleeping time or aminopyrine demethylase activity. When this interval was shortened, we could not demonstrate a significant decrease in hexobarbital sleeping time or an increase in aminopyrine demethylase activity. We could not demonstrate increased tolerance to a toxic dose of methoxyflurane following pretreatment unless the interval between pretreatment and exposure to the lethal concentration was at least 96 hours. These observations suggest that methoxyflurane may exert a biphasic effect on drug metabolism, that is, it first inhibits and then stimulates drug metabolism after 12 hours.$^{2}$ The apparent delay before stimulation of drug metabolism by methoxyflurane may represent the time required to synthesize enzymic protein, and/or time required to eliminate methoxyflurane.

The behavior of rats exposed to a subanesthetic concentration of methoxyflurane as used in this study could not be differentiated from that of control rats. The possibility that hypoxia and/or hypercarbia occurred during pretreatment with the subanesthetic concentration of the methoxyflurane used in this study, while unlikely, cannot be ruled out. The effects on drug metabolism of chronic exposure to hypoxia and hypercarbia are under investigation.

The screening test to determine if ip administration of BHT affected microsomal enzymic activity indicated that fairly large doses of BHT were necessary for demonstration of a decrease in hexobarbital sleeping time. The weights of the rats at the beginning of the experiment were between 40 to 50 g; weights (control and treated) at the end of the experiment averaged 90 g. The dose of BHT which markedly shortened the hexobarbital sleeping time was 10 mg twice a day per rat. Converting this dose to a kilogram body weight basis, it would be 200 to 250 mg administered twice a day at the beginning of the experiment and 11.5 mg twice a day at the end. It is extremely doubtful that BHT would volatilize under the conditions used to vaporize methoxyflurane in this study (personal communication, E. R. Larson, Dow Chemical Company), so it can be presumed that rats exposed to methoxyflurane did not inhale an amount of BHT sufficient to affect drug metabolism.

<table>
<thead>
<tr>
<th>Pretreatment*</th>
<th>9,000 X g Protein mg/g Wet Weight of Liver (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>636.1 ± 49.52</td>
</tr>
<tr>
<td>Methoxyflurane, 0.13 per cent, 7 hours/day × 10 days</td>
<td>799.1 ± 60.62†</td>
</tr>
</tbody>
</table>

* Six assays per treatment. Each assay represents the livers from three rats.
† P < 0.001.
The authors thank Abbott Laboratories for their generous supplies of methoxyflurane and butylated hydroxytoluene.

References