The Effects of Forane and Flurxene on Mitochondrial Respiration:
Correlation with Lipid Solubility and In-vivo Potency

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Rat liver mitochondria were exposed to various concentrations of Forane and fluroxene. Polarographic measurement of oxygen uptake during glutamate oxidation demonstrated dose-related reversible inhibition of state 3 respiration following treatment with each agent. In contrast, state 3 succinate oxidation remained unaffected, although irreversible loss of respiratory control followed exposure to high anesthetic concentrations. Thus, Forane and fluroxene interfere with glutamate oxidation by blocking electron transport at the NADH dehydrogenase locus in a fashion similar to other volatile anesthetics.

Inhibition of state 3 glutamate oxidation by rat liver mitochondria has been demonstrated previously for halothane, diethyl ether, methoxyflurane and enflurane. The concentrations of anesthetic necessary for 50 per cent inhibition of state 3 respiration (ID₅₀) were calculated for all six agents and found to bear an inverse log-log relationship to lipid solubility. Potency in vitro (ID₅₀) was directly related to potency in vivo (MAC). This relationship may reflect similar molecular mechanisms of action. (Key words: Forane; Flurxene; Mitochondria; Respiration; Lipid solubility; Anesthetic potency.)

HALOTHANE, methoxyflurane, enflurane (Etherane, 2-chloro-1,1,2-trifluoroethyl difluoromethyl ether), and diethyl ether have been shown to inhibit state 3 oxygen uptake by rat liver mitochondria in the presence of nicotinamide adenine dinucleotide (NAD)-linked substrates. Oxidation of succinate (a non-NAD-linked substrate) is affected considerably less than that of glutamate, and it is thought that all four agents interfere with glutamate utilization by blocking oxidation of reduced nicotinamide adenine dinucleotide (NADH).¹ ² ³

Forane (1-chloro-2,2,3-trifluoroethyl difluoromethyl ether) might be expected to affect mitochondrial respiration in a fashion similar to that of enflurane, its isomer. In contrast, fluroxene has shown little effect on mitochondrial calcium uptake ⁴ and thus may not affect other mitochondrial functions. Accordingly, the present study was designed to assess the effects of Forane and fluroxene on state 3 and state 4 mitochondrial oxygen uptake utilizing NADH-linked and non-NADH-linked substrates.

The relationship between lipid solubility of an inhalation anesthetic and the minimum alveolar concentration necessary to prevent gross movement in response to surgical incision in 50 per cent of patients (MAC) is well established.⁵ Data obtained using halothane, methoxyflurane, diethyl ether and enflurane have suggested a similar relationship between lipid solubility and the concentration of anesthetic necessary to inhibit state 3 glutamate oxidation in rat liver mitochondria by 50 per cent (ID₅₀).¹ ² Elucidation of the effects of Forane and fluroxene on mitochondrial respiration would bring the total number of anesthetics studied to six and enable more accurate comparison between in-vitro potency (ID₅₀) and lipid solubility.

Methods

Chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Hepatic mitochondria were prepared by a modification of the method of Schneider,⁶ with all operations being carried out at 0–4°C. Male or female Wistar rats weighing 180–200 g were sacrificed by decapitation and the livers immediately removed and minced in cold isotonic homogenizing medium consisting of 0.225 m

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mannitol, 0.075 M sucrose, and 200 μM EDTA at pH 7. A uniform homogenate was prepared by grinding the tissue in a teflon pestle homogenizer (Thomas 3431E25) driven at 890 rpm. The homogenate was centrifuged at 500 × g for 10 minutes to remove broken cells, connective tissue, and erythrocytes. The resulting supernate was centrifuged at 12,500 × g for 10 minutes to sediment the mitochondria. The mitochondrial pellet was washed twice by resuspending in homogenizing medium and centrifuging 10 minutes at 12,500 × g. The final mitochondrial pellet was resuspended in 4–7 ml homogenizing medium and, contained 22–51 mg protein per ml as assayed by the Biuret method.

Samples of mitochondrial suspension (0.2–0.5 ml) were placed in each of two 50-ml Florence flasks attached to a Burrell flask shaker and suspended in an ice bath. The circuit for exposing mitochondria to Forane and fluoroxene is diagrammed in Figure 1. The anesthetics were vaporized with a 1-1/min flow of air through a Dräger halothane vaporizer, or with various flows through a gas-washing bottle (Thomas 5632-F40) maintained in a constant-temperature water bath. In the latter instance, saturated anesthetic vapor was diluted with air to provide the desired concentration. Anesthetic vapor at a flow of 20 ml/min was directed to one flask (anesthetized), while the second (control) flask received 20 ml/min air. The suspensions were equilibrated for 20 minutes, and gently shaken for the first and last five minutes of this period. Samples of mitochondria were then removed from each flask and immediately assayed for oxygen uptake. To assess the reversibility of any anesthetic effect, the vaporizer was turned off and mitochondria remaining in the anesthetized flask treated with a 20 ml/min flow of air for 20 minutes, again with two periods of shaking, a sample of these "resuscitated" mitochondria was assayed for oxygen uptake. Anesthetic concentrations, determined by gas chromatography and expressed as per cent of a standard atmosphere, ranged from 0 to 5.99 per cent for Forane and from 0 to 26.56 per cent for fluoroxene. Concentration was measured each time a preparation was anesthetized, except when Forane was tested in the presence of glutamate. A gas chromatograph was not available at that time, so the Dräger vaporizer was subsequently calibrated under the experimental conditions outlined above. There was a linear relationship between delivered concentration (DC), expressed as per cent of a standard atmosphere, and indicated concentration (IC) such that DC = 1.14 IC + 0.22 (r = 0.986, P < 0.001; F = 471, P < 0.001). Forane concentrations in Figure 2 refer to concentrations delivered by the vaporizer.

Oxygen uptake was measured polarographically with a vibrating platinum electrode (Gilson oxygraph) at 25 C. Prior to addition of mitochondrial suspension, the cuvette was filled with 1.6 ml reaction medium (0.157 M mannitol, 0.062 M sucrose, 0.010 M Trizma base, and 0.010 M KH₂PO₄ at pH 7.2) and 0.2 ml 0.1 M glutamate or 1.7 ml reaction medium and 0.2 ml 0.1 M succinate. Air or anesthetic in air was then bubbled through the cuvette at 20 ml/min. Initial oxygen concentration was calculated from measured barometric pressure and the known solubility of oxygen in the reaction medium. Corrections were made for the presence of anesthetics as necessary. Mitochondrial suspension (0.2 ml during glutamate experiments or 0.1 ml during succinate experiments) was then added to the cuvette and oxygen uptake was measured both in the absence (state 4) and in the presence (state 3) of exogenous adenosine diphosphate (ADP). ADP was added to a final concentration of 159–224 μM. Oxygen uptake was expressed as nmol/min/mg mitochondrial protein. ADP concentrations were determined spectrophotometrically.

Results

The effect of Forane on oxygen uptake by rat liver mitochondria when glutamate was substrate is shown in Figure 2. State 3 respiration was depressed 15 per cent after treatment with 1.4 per cent Forane. Higher concentrations of anesthetic progressively diminished state 3 uptake until there was virtually no response to ADP with 5.8 per cent Forane. After treatment with air for 20 minutes, recovery was complete, even in preparations exposed to concentrations of anesthetic which depressed state 3 respiration to 40 per cent of control values.
The term "respiratory control" denotes the slow rate of mitochondrial respiration when ADP is absent and oxygen and substrate are in excess, i.e., state 4. Treatment of mitochondrial suspensions with Forane did not diminish state 3 succinate oxidation (table 1), and thus respiratory rate during state 4 serves as an index of respiratory control. Exposure to as much as 4.3 per cent Forane had no effect on state 4. However, treatment with higher concentrations resulted in nearly complete and irreversible loss of respiratory control.

Fluroxene's effect on glutamate oxidation is documented in figure 3. As with Forane, there was dose-dependent inhibition when glutamate was substrate. Reversibility was present with as much as 24.5 per cent fluroxene. Control values obtained when fluroxene was tested in the presence of glutamate were somewhat lower than those obtained in other experiments. The use of a specific substrain of rat (Lewis) in this instance may account for the apparent discrepancy.

When succinate was substrate, fluroxene also had little effect on state 3 respiration (table 2). Oxygen uptake during state 4 increased over control with concentrations of fluroxene greater than 2.8 per cent and states 3 and 4 proceeded at the same rate after exposure to 9.9 per cent. Reversibility could not be clearly demonstrated at any concentration studied.

**Discussion**

Several difficulties are inherent in assessing the effects of anesthetics on mitochondrial respiration. An in-vitro system must be used for measurement of oxygen uptake; rat liver mitochondria were chosen for ease of preparation and reproducibility of control values. Exposure to anesthetics might be accomplished by anesthetizing intact animals and subsequently preparing mitochondria. However, this results in loss of anesthetic vapor if mitochondria are isolated in anesthetic-free media. Thus, intact mitochondria were exposed directly to anesthetic vapor. Exposure to vapor rather than solutions of liquid anesthetic provides ease of calculation of anesthetic concentration, avoids volatile impurities in commercial preparations of liquid anesthetic, and has resulted in reproducible data in previous studies. Loss of anesthetic into the reaction medium during oxygen uptake determinations was minimized by prior equilibration of the medium with anesthetic. Equilibration at 0–4 C.
and measurement of oxygen uptake at 25°C were necessary because exposure to body temperature results in loss of respiratory control in untreated preparations.

Previous studies from this laboratory have shown that when glutamate or NADH is provided as substrate, state 3 respiration by intact mitochondria is inhibited by halothane, enflurane, methoxyflurane, and diethyl ether. State 3 succinate oxidation is unaffected by halothane and enflurane, while methoxyflurane and diethyl ether produce a rather small de-

**Table 1. Effects of Forane on Oxygen Uptake and Respiratory Control of Rat Liver Mitochondria**

<table>
<thead>
<tr>
<th>Forane Concentration (Per Cent)</th>
<th>Oxygen Uptake (nmol/min/mg Protein)</th>
<th>Anesthetized</th>
<th>“Resuscitated”</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>State 3</td>
<td>State 4</td>
</tr>
<tr>
<td>None (control)</td>
<td></td>
<td>43.08 ± 2.39</td>
<td>13.25 ± 0.82</td>
</tr>
<tr>
<td>1.02 ± 0.06</td>
<td></td>
<td>39.67 ± 5.24</td>
<td>13.67 ± 1.76</td>
</tr>
<tr>
<td>2.55 ± 0.13</td>
<td></td>
<td>41.00 ± 4.51</td>
<td>13.33 ± 1.76</td>
</tr>
<tr>
<td>4.33 ± 0.06</td>
<td></td>
<td>41.67 ± 8.17</td>
<td>13.33 ± 1.76</td>
</tr>
<tr>
<td>5.71 ± 0.14</td>
<td></td>
<td>51.00 ± 7.00</td>
<td>43.00 ± 10.41</td>
</tr>
</tbody>
</table>

* The substrate was succinate. Values are means ± SE.
Fig. 3. Effect of fluroxene on state 3 oxygen uptake of rat liver mitochondria. The substrate was glutamate (means ± SE).

gree of inhibition.\textsuperscript{2,3} Thus, all four anesthetics are thought to affect glutamate oxidation by blocking NADH dehydrogenase.

No previous data concerning the effects of Forane on mitochondrial function are available. However, the present study has demonstrated dose-related inhibition of state 3 glutamate oxidation similar to that produced by enflurane, its isomer. This effect was evident after exposure to clinically useful concentrations and was readily reversible. Succinate oxidation was inhibited only after treatment with high doses and there was resultant irreversible loss of respiratory control.

**Table 2. Effects of Fluroxene on Oxygen Uptake and Respiratory Control of Rat Liver Mitochondria**

<table>
<thead>
<tr>
<th>Fluroxene Concentration (Per Cent)</th>
<th>Oxygen Uptake (nmol/min/mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anesthetized</td>
</tr>
<tr>
<td></td>
<td>State 3</td>
</tr>
<tr>
<td>None (control)</td>
<td>42.10 ± 1.09</td>
</tr>
<tr>
<td>11.68 ± 0.68</td>
<td>41.33 ± 3.93</td>
</tr>
<tr>
<td>2.79 ± 0.36</td>
<td>51.67 ± 3.18</td>
</tr>
<tr>
<td>† 5.47 ± 0.40</td>
<td>42.00 ± 0.58</td>
</tr>
<tr>
<td>7.32 ± 0.16</td>
<td>41.75 ± 4.37</td>
</tr>
<tr>
<td>9.58 ± 0.21</td>
<td>47.88 ± 3.31</td>
</tr>
</tbody>
</table>

* The substrate was succinate. Values are means ± SE.
† Because of condensation of fluroxene vapor at this concentration, resuscitation was not attempted.
TABLE 3. Anesthetic Potencies and Lipid Solubilities of Six Anesthetics

<table>
<thead>
<tr>
<th></th>
<th>ID₅₀ (Per Cent)</th>
<th>Oil/Gas Partition Coefficient at 37 °C</th>
<th>MAC* (Per Cent)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoxene</td>
<td>18.88</td>
<td>48</td>
<td>3.4</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>7.61</td>
<td>65</td>
<td>1.92</td>
</tr>
<tr>
<td>Enflurane</td>
<td>2.92</td>
<td>95</td>
<td>1.68</td>
</tr>
<tr>
<td>Forane</td>
<td>4.96</td>
<td>99</td>
<td>1.28</td>
</tr>
<tr>
<td>Halothane</td>
<td>1.45</td>
<td>221</td>
<td>0.77</td>
</tr>
<tr>
<td>Methoxyflurane</td>
<td>0.62</td>
<td>970</td>
<td>0.16</td>
</tr>
</tbody>
</table>

* Values were obtained from references 5, 17-19.

Although chloroform and halothane inhibit calcium uptake by intact mitochondria, fluoxene has no effect. Thus, it was thought that other mitochondrial functions might remain intact after exposure to fluoxene. However, inhibition of state 3 glutamate oxidation was evident after treatment with as little as 2.6 per cent fluoxene, and high concentrations produced profound depression. State 3 succinate oxidation was unaffected by as much as 9.9 per cent fluoxene, but loss of respiratory control occurred after exposure to 2.8 per cent. These data confirm the work of Harris et al., which showed inhibition of NADH oxidase after treatment of mitochondrial electron transport particles with fluoxene. They found no inhibition of succinate oxidation.

Thus, the effects of Forane and fluoxene on mitochondrial respiration are similar to those of other volatile anesthetics. Electron transport is blocked in dose-related fashion at the NADH dehydrogenase locus. Direct extrapolation of these in vitro findings to intact

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**Fig. 4.** Relationship between in vitro potency and lipid solubility.

\[
\log \text{ID}_{50} = 2.746 - 1.035 \log O'/G \\
\text{r} = 0.929 \quad (p<0.01) \\
\text{SE}_{\text{slope}} = 0.206 \quad (p<0.01) \\
\text{F} = 25.162 \quad (p<0.01)
\]
organisms undergoing clinical anesthesia is not possible. However, evidence for a relationship between potency in vitro and lipid solubility is strengthened by the data of the present study.

Diethyl ether,\textsuperscript{2} Forane, and fluroxene linearly diminished state 3 oxygen uptake over the entire range of anesthetic concentrations studied. When individual data for each of these anesthetics were subjected to regression analysis by the method of least squares,\textsuperscript{5} a linear equation relating anesthetic concentration to oxygen uptake is obtained. ID\textsubscript{50} values can then be derived by solving the individual regression equations for the concentration of anesthetic necessary to inhibit oxygen uptake by 50 per cent.

Halothane, methoxyflurane, and enfurane also linearly depress state 3 oxygen uptake, but a point at which higher concentrations of anesthetic have no further effect is reached.\textsuperscript{1,2} For halothane, this phenomenon has been shown to result from lack of inhibition of the amytal-insensitive pathway of dihydrornicotinamide adenine dinucleotide metabolism.\textsuperscript{14, 15, 16}

Therefore, in calculating the ID\textsubscript{50} values for these agents, only individual data on the linear portions of the dose-response curves are included. For each of the six agents, the ID\textsubscript{50} is contained in the range of anesthetic concentrations used for its calculation.

Individual values for ID\textsubscript{50} and oil/gas partition coefficient (O/G) appear in table 3. The logarithmic relationship of ID\textsubscript{50} to O/G can be examined by regression analysis.\textsuperscript{8} It is significant at the 1 per cent level (fig. 4).

Eger and co-workers suggested that the best correlation of anesthetic potency with a physical property was obtained using lipid solubility as defined by O/G.\textsuperscript{5} To permit comparison with the present study, Eger’s data (table 3) were subjected to regression analysis, which yielded the equation:

\[
\log \text{MAC} = 2.105 - 0.969 \log \text{O/G}
\]

\[r = 0.998, \ P < 0.001; \ SE_{\text{slope}} = 0.026, \ P <\]

![Graph showing the relationship between ID\textsubscript{50} and MAC for different agents.](image)

**Fig. 5.** Relationship between potency in vivo and in vitro.
The slope of this equation does not differ significantly from that of the equation relating to \( ID_{50} \) to lipid solubility:

\[
\log ID_{50} = 2.746 - 1.035 \log O/G
\]

\( r = 0.929, \ P < 0.01; \ SE_{\text{slope}} = 0.206, \ P < 0.01; \ F = 25, \ P < 0.01. \)

Thus, mathematical considerations would predict the linear correlation between \( ID_{50} \) and MAC which is illustrated in figure 5 and described by the equation:

\[
ID_{50} = 5.703 \text{ MAC} - 2.733
\]

\( r = 0.934, \ P < 0.01; \ SE_{\text{slope}} = 1.088; \ P < 0.01; \ F = 27, \ P < 0.01. \)

The difference between MAC and \( ID_{50} \) can be attributed to several factors. MAC is referable to the central nervous system in man, while \( ID_{50} \) is measured in isolated rat liver mitochondria. The molecular events reflected by MAC occur in a "physiologic" environment in the presence of many substrates. Mitochondria are suspended in an isotonic sucrose-mannitol medium and provided only with oxygen, inorganic phosphate, ADP, and glutamate. MAC is measured at 37°C, while mitochondria are exposed to anesthetics at 0–4°C and oxygen uptake is measured at 25°C.

Thus, at present it is not suggested that the anesthetic state results from depression of mitochondrial function. However, the striking similarity of the relationships of in vivo potency and in vitro potency to lipid solubility suggests that a common process might be involved in the production of narcosis and inhibition of mitochondrial respiration. Further elucidation of the subcellular mechanisms responsible may help to explain the molecular basis for narcosis.

Forane was a gift of Ohio Chemical Products, a Division of Air Reduction Co., Inc.

References


4. Miller RN: Personal communication


