The effect of fluroxene [(2,2,2-trifluoroethoxy)ethane] on haem biosynthesis and degradation

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Acute fluroxene treatment of male Wistar rats decreases the amounts of hepatic microsomal cytochrome P-450 and haem, increases the activities of hepatic δ-aminolaevulinate synthase and haem oxygenase, and increases the amounts of haem precursors (δ-aminolaevulinate and porphobilinogen) in the urine. All of the above effects of fluroxene are enhanced by pretreatment of the experimental animals with 3-methylcholanthrene and phenobarbital. The amounts of porphyrins in the urine and faeces were generally unaffected by acute fluroxene treatment of uninduced or 3-methylcholanthrene- or phenobarbital-induced Wistar rats. 2,2,2-Trifluoroethyl ethyl ether, the saturated analogue of fluroxene, did not affect the amounts of hepatic cytochrome P-450 and haem, the amounts of any of the haem precursors in the urine or faeces, or the activity of hepatic haem oxygenase in phenobarbital-induced male Wistar rats. The amounts of hepatic cytochrome P-450 and haem and of the haem precursors in urine and faeces, and the activity of δ-aminolaevulinate synthase, were generally not altered by acute fluroxene treatment of uninduced male Long–Evans rats. Chronic treatment of Wistar rats with fluroxene resulted in small increases in the amounts of δ-aminolaevulinate and porphyrins in urine. The amounts of porphobilinogen in urine were elevated up to 2000%, whereas the amounts of the porphyrins in faeces were generally unaffected. After chronic fluroxene treatment, the activity of δ-aminolaevulinate synthase was increased, whereas the activity of uroporphyrinogen synthase was decreased. It is concluded that acute fluroxene treatment may affect haem biosynthesis and degradation by a mechanism similar to allylisopropylacetamide, namely by stimulating an atypical cytochrome P-450-dependent pathway for haem degradation. The effects of chronic fluroxene treatment on haem biosynthesis may be a consequence of this mechanism or a result of the inhibition by fluroxene of uroporphyrinogen synthase. Chronic fluroxene treatment of male rats affects the haem biosynthetic pathway in a manner similar to that seen in human genetic acute intermittent porphyria.

Fluroxene is a volatile anaesthetic agent that was utilized in clinical practice between 1953 and 1977. The first step in the metabolism of fluroxene in vivo and in vitro is catalysed by hepatic microsomal cytochrome P-450 (see, e.g., Ivanetich et al., 1976b). The metabolism of fluroxene by cytochrome P-450 results in a decrease in the amount and activity of hepatic microsomal cytochrome P-450 that appears to reflect the chemical modification of the haem moiety of the enzyme by a transient reactive metabolite of fluroxene (Ivanetich et al., 1975; Marsh et al., 1977; Ivanetich & Bradshaw, 1977; Bradshaw et al., 1978). 2,2,2-Trifluoroethyl ethyl ether, the saturated analogue of fluroxene, does not alter the haem of cytochrome P-450 in vitro or in vivo (Ivanetich et al., 1975, 1976a).

The degradation of the haem of cytochrome P-450 by fluroxene is similar in many respects to the degradation of the haem of this enzyme by allylisopropylacetamide. Both compounds require prior
metabolic activation, apparently by cytochrome P-450, in order to degrade hepatic microsomal cytochrome P-450. The mechanisms of action of fluoxene and allylisopropylacetamide do not involve lipid peroxidation or the normal physiological pathway for haem degradation via haem oxygenase (Landaw et al., 1970; De Matteis, 1973; Levin et al., 1973; Ivanetich et al., 1975; Marsh et al., 1977; De Matteis et al., 1977). With both fluoxene and allylisopropylacetamide, the haem of cytochrome P-450 is converted into green pigments that appear to be adducts of an activated form of the compounds with a vinyl side chain of the haem moiety (Ortiz de Montellano, 1978ab; J. J. Bradshaw & K. M. Ivanetich, unpublished work).

The degradation of the haem of cytochrome P-450 by allylisopropylacetamide is thought to play a major role in the enhancement by allylisopropylacetamide of the rate of haem biosynthesis and in the production by allylisopropylacetamide of experimental porphyria in laboratory rodents (De Matteis, 1973; Bissell & Hammermaker, 1976b). Allylisopropylacetamide is thought to act as follows. Firstly, allylisopropylacetamide degrades the haem of cytochrome P-450, then the apoprotein of this enzyme draws haem from a haem pool, which is thought to regulate haem biosynthesis and degradation (De Matteis, 1971; Meyer & Schmid, 1973; Tschudy & Bonkowsky, 1972). The decreased amounts of haem in the pool result in decreased negative-feedback control by haem and the stimulation of the synthesis of δ-aminolaevulinate synthase and subsequent increases in the rate of synthesis of the haem precursors, i.e. δ-aminolaevulinate, porphobilinogen and the porphyrins (Schmid & Schwartz, 1952; Goldberg et al., 1955; Schmid et al., 1955; Bissell & Hammermaker, 1976b).

The effects of allylisopropylacetamide on haem biosynthesis, namely the induction of δ-aminolaevulinate synthase and the increased amounts of haem precursors in the urine, are characteristic of a group of human diseases known as the porphyrias (see, e.g., Meyer & Schmid, 1977). The particular type of genetic human porphyria that allylisopropylacetamide mimics in laboratory rodents is known as acute intermittent porphyria (Goldberg et al., 1955; Tschudy & Bonkowsky, 1972).

Since the characteristics of the degradation of the haem of cytochrome P-450 by fluoxene and allylisopropylacetamide are similar in so many respects, it was thought that fluoxene might affect haem biosynthesis in experimental animals by virtue of its ability to degrade the haem of hepatic microsomal cytochrome P-450 in vivo. For this reason, the effects of fluoxene on hepatic haem biosynthesis and degradation in experimental animals have been investigated and are reported in the present paper.

Materials and methods

Materials

Fluoxene was obtained from Ohio Medical Products, Madison, WI, U.S.A. Sodium phenobarbital and 3-methylcholanthrene were supplied by Maybaker Ltd., Port Elizabeth, South Africa, and Eastman Kodak, Rochester, NY, U.S.A. respectively. NADPH and crystalline bovine serum albumin were purchased from Miles Laboratories, Cape Town, South Africa. Haemin (haematin hydrochloride) was obtained from BDH Ltd., Poole, Dorset, U.K. δ-Aminolaevulinate hydrochloride and porphobilinogen were supplied by Sigma Chemical Company, St. Louis, MO, U.S.A. Cyclohexanone and acetylacetone were obtained from Merck Chemicals, Darmstadt, Germany. Afrox Limited, Cape Town, South Africa, supplied cylinders of pure compressed gases. 2,2,2-Trifluoroethyl ethyl ether was prepared by the hydrogenation of fluoxene as described by Ivanetich et al. (1975). All other chemicals used were of the highest purity commercially available. Water was glass-distilled and deionized.

Treatment of animals

Male Wistar rats weighing between 190 and 210g were used for all experiments unless otherwise stated. In the latter case, male Long–Evans rats weighing between 190 and 210g were used. The animals were allowed free access to Epol laboratory chow (protein minimum 20%, fat 2.5%, fibre maximum 6%, calcium 1.4%, phosphorus 0.7%) unless otherwise indicated, and were allowed free access to water at all times.

Cytochrome P-450 was induced by intraperitoneal injection of sodium phenobarbital [80 mg per kg in 0.9% (w/v) NaCl for 1 day] or 3-methylcholanthrene (40 mg/day per kg in corn oil for 3 consecutive days). Animals that had been induced with sodium phenobarbital were administered fluoxene at a dose of 1 ml/kg†, whereas animals pretreated with 3-methylcholanthrene received 4 ml/kg† of fluoxene. Uninduced animals received either 4 ml/kg or 6 ml/kg† of fluoxene. To both the 3-methylcholanthrene- and phenobarbital-pretreated animals, fluoxene was administered 24h after the last injection of inducing agent. In experiments where animals were chronically exposed to fluoxene, a dose of 1 ml/kg† was administered to uninduced rats at 48h intervals. In all cases fluoxene was administered by intraperitoneal injection. 2,2,2-Trifluoroethyl ethyl ether was injected intraperitoneally into phenobarbital-pretreated rats at a dose of 1 ml/kg.

† These doses of fluoxene represent the maximum doses that could be administered to the Wistar rats without fatality.
Fluroxene and haem metabolism

Animals were normally starved for 24 h after the last injection of fluroxene, and then killed immediately by cervical fracture. For determination of the activities of δ-aminolaevulinate synthase and uroporphyrinogen synthase, the rats were killed 16 h after the final injection of fluroxene or at an equivalent time in control rats.

Collection of urine and faeces

Immediately after fluroxene treatment, animals were housed in groups of three in perspex metabolic cages for the collection of urine and faeces. Animals were starved but allowed water *ad libitum* over the 24 h collection period. In the experiments where fluroxene was administered over a period of 2–3 weeks, the animals were allowed free access to food until 24 h before being killed and free access to water throughout the experiment. In these experiments, the urine and faeces were collected over periods of 48 h.

Quantification of haem precursors

The colorimetric method of Mauzerall & Granick (1956) was used for the quantitative determination of δ-aminolaevulinate and porphobilinogen in urine. The method of Schwartz et al. (1951) as modified by Holti et al. (1958) was used to determine coproporphyrin in urine, except that initially 5 ml of urine was shaken with 0.5 ml of acetic acid plus 20 ml of diethyl ether, and subsequently the combined ether extracts were washed once each with 20 ml of 0.5% (w/v) sodium acetate, 20 ml of 0.005% (w/v) iodine and 10 ml of water. The correction formula of Rimington & Sveinsson (1950) as modified by With (1955) was used to calculate the amount of coproporphyrin in urine, utilizing the absorption coefficient given by Fuhrhop & Smith (1975). Uroporphyrin in urine was determined spectrally as described by Dresel et al. (1956), by using the correction formula described by Rimington & Sveinsson (1950).

Coproporphyrin and protoporphyrin in faeces were determined by the method of Holti et al. (1958) with the following minor modifications. The combined ether extracts of the dried faeces were extracted once each with 20 ml of saturated sodium acetate, 20 ml of 0.005% (w/v) iodine and 20 ml of water. After the coproporphyrin was extracted from the ether into 0.1 M-HCl, the protoporphyrin was repeatedly extracted with 1.5 M-HCl. The correction formulas described by Rimington & Sveinsson (1950) as modified by With (1955) were used for calculation of the coproporphyrin and protoporphyrin content of the faeces.

Liver enzyme assays

Hepatic microsomal fraction and postmitochondrial supernatant were prepared from rat liver homogenates at 4°C by differential ultracentrifugation as described by Holtzman & Carr (1972). The microsomal fraction was finally suspended at a concentration of 2 mg of protein/ml in 0.02 M-Tris/HCl, pH 7.4, and was used immediately after preparation. The protein concentration was determined by the method of Lowry et al. (1951) as modified by Chaykin (1966), with bovine serum albumin as a standard.

The concentrations of hepatic microsomal cytochrome P-450 and haem were determined by the method of Omura & Sato (1964a,b).

The method of Tenhunen et al. (1969) was used with minor modifications to determine hepatic haem oxygenase activity. The sample cuvette for the assay contained liver postmitochondrial (18 000 g) supernatant (approx. 1.5 mg of protein/ml), 17 μM-haemin, 0.6 mM-NADPH and 90 mM-potassium phosphate buffer, pH 7.4. The reference cuvette contained the above components, except that NADPH was replaced by an equivalent volume of 90 mM-potassium phosphate buffer, pH 7.4. The formation of bilirubin was determined from the increase in \( A_{468} \) (ε468 = 30 mm⁻¹·cm⁻¹).

The activity of uroporphyrinogen synthetase in the postmitochondrial supernatant was determined by monitoring the loss of porphobilinogen, as described by Hutton & Gross (1970). The method of Marver et al. (1966a,b) was utilized for the determination of δ-aminolaevulinate synthase activity in rat liver homogenates.

Statistical analysis

Results are given as means ± S.D. Determinations on each day were on the pooled livers, urine or faeces of three to four treated and three to four control rats. Assays were performed in duplicate, triplicate or quadruplicate, except for the determinations of the porphyrins in urine and faeces, where sample size permitted single determinations only.

Statistical analysis of the data was performed with the two-tailed Student’s *t* test for unpaired data. *P* values below 0.05 were regarded as being probably significant, with *P* values below 0.01 being significant and *P* values below 0.001 being highly significant.

Spectrophotometry

Unless otherwise stated, a Unicam SP. 1800 spectrophotometer coupled to a Unicam AR 25 recorder was used for all spectral measurements. The compartment adjacent to the photomultiplier was used for particulate samples.

Results

**Effect of acute fluroxene treatment on hepatic microsomal enzyme activities and haem content**

The effect of acute fluroxene treatment on the
or amounts of cytochrome P-450 and haem and activity of haem oxygenase was determined with uninduced and induced rats (Table 1). Single injections of fluroxene (4 ml/kg) into uninduced Wistar rats resulted in significant decreases in the amounts of hepatic microsomal cytochrome P-450. The extent of loss of cytochrome P-450 was greater in rats given a higher dose of fluroxene and in rats that had been induced with phenobarbital or 3-methylcholanthrene. In all cases the losses of cytochrome P-450 were accompanied by similar losses of microsomal haem. The amounts of cytochrome P-450 and haem in Long–Evans rats were not significantly affected by fluroxene (Table 1).

Significant increases in the activity of hepatic microsomal haem oxygenase were observed after fluroxene treatment (4 ml/kg) of uninduced Wistar rats. Greater increases in the activity of haem oxygenase were seen at higher doses of fluroxene or in rats previously induced with phenobarbital or 3-methylcholanthrene (Table 1). In Long–Evans rats, fluroxene did not significantly affect the activity of haem oxygenase. In general, there appears to be a direct relationship between the extent of degradation of hepatic microsomal cytochrome P-450 and haem and the activity of hepatic haem oxygenase (Table 1).

In fluroxene-treated phenobarbital-induced rats, the content of cytochrome P-450 decreased before the activity of haem oxygenase increased (Table 2).}

**Effect of acute fluroxene treatment on hepatic haem biosynthesis**

Single injections of fluroxene to Wistar rats resulted in slight, but not significant, increases in the amounts of δ-aminolevulinate in uninduced rats. Significantly increased amounts of δ-aminolevulinate did, however, follow fluroxene treatment of Wistar rats previously induced with 3-methylcholanthrene or phenobarbital (Table 3). Porphobilinogen was significantly elevated by fluroxene in both uninduced and phenobarbital- or 3-methylcholanthrene-induced Wistar rats, with the increases in the amounts of porphobilinogen excreted being of the greatest magnitude in 3-methylcholanthrene-induced rats. No significant changes in the amounts of porphyrin in urine and faeces were observed after fluroxene treatment of uninduced and induced rats. No significant changes in the amounts of haem precursors in urine and faeces were observed in Long–Evans rats treated with fluroxene, except for a significant increase in the amount of δ-aminolevulinate in urine (Table 3).

Both uninduced and induced Wistar rats exhibited significant increases in the activity of δ-aminolevulinate synthase after fluroxene treatment (Table 3). The most striking increase in the activity of δ-aminolevulinate synthase was observed after fluroxene treatment of 3-methylcholanthrene-induced rats.

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**Table 1. Effect of fluroxene on the amounts of hepatic microsomal cytochrome P-450 and haem and the activity of haem oxygenase in induced and uninduced rats**

Male Wistar rats were pretreated with inducing agents and injected with fluroxene as described in the Materials and methods section. The rats were killed 24 h after the fluroxene injection or at an equivalent time in rats not treated with fluroxene. Assays were performed on hepatic microsomal fractions or postmitochondrial supernatant as described in the Materials and methods section. Results are means ± S.D. for assays performed in triplicate or more on each of three or four separate groups of three or more animals.

<table>
<thead>
<tr>
<th>Inducing agent</th>
<th>Dosage of fluroxene (ml/kg)</th>
<th>Loss/initial values (nmol/mg of microsomal protein)</th>
<th>Amount remaining after fluroxene treatment (%)</th>
<th>Loss/initial values (nmol/mg of microsomal protein)</th>
<th>Fluroxene treated/untreated rats (nmol of bilirubin/10 min per mg of protein)</th>
<th>Activity relative to controls (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
<td>4.0</td>
<td>0.09/(0.83 ± 0.04)</td>
<td>89</td>
<td>0.08/(1.47 ± 0.05)</td>
<td>0.19 ± 0.12/0.07 ± 0.01†</td>
<td>271</td>
</tr>
<tr>
<td>None</td>
<td>4.0</td>
<td>0.29/(1.17 ± 0.19)</td>
<td>75†</td>
<td>0.12/(1.58 ± 0.21)</td>
<td>0.23 ± 0.13/0.07 ± 0.04‡</td>
<td>329</td>
</tr>
<tr>
<td>None</td>
<td>6.0</td>
<td>0.37/(0.85 ± 0.14)</td>
<td>56‡</td>
<td>0.43/(1.49 ± 0.13)</td>
<td>0.68 ± 0.29/0.09 ± 0.03§</td>
<td>756</td>
</tr>
<tr>
<td>3-Methyl-</td>
<td>4.0</td>
<td>0.88/(1.66 ± 0.45)</td>
<td>53§</td>
<td>0.72/(2.63 ± 0.43)</td>
<td>0.40 ± 0.07/0.07 ± 0.05§</td>
<td>571</td>
</tr>
<tr>
<td>Cholanthrene</td>
<td>1.0</td>
<td>1.25/(1.98 ± 0.08)</td>
<td>37§</td>
<td>1.16/(2.76 ± 0.08)</td>
<td>0.51 ± 0.09/0.06 ± 0.01§</td>
<td>850</td>
</tr>
</tbody>
</table>

† Probably differs from identically induced rats not treated with fluroxene, P < 0.05.
‡ Differs significantly from identically induced rats not treated with fluroxene, P < 0.01.
§ Differs significantly from identically induced rats not treated with fluroxene, P < 0.001.
**Effect of acute 2,2,2-trifluoroethyl ethyl ether treatment on the amounts of hepatic cytochrome P-450 and haem and on haem metabolism**

The effect of a single injection of 2,2,2-trifluoroethyl ethyl ether on the activities or amounts of hepatic microsomal enzymes and haem and on haem metabolism was assessed in phenobarbital-induced Wistar rats. As was found previously for 2,2,2-trifluoroethyl ethyl ether anaesthesia (Ivanetich et al., 1976a), intraperitoneal injection of 2,2,2-trifluoroethyl ethyl ether into phenobarbital-induced rats had no effect on the amounts of hepatic microsomal cytochrome P-450 and haem. The concentrations of cytochrome P-450 were 2.04 ± 0.02 and 1.95 ± 0.01 nmol/mg of microsomal protein and of haem were 2.18 ± 0.09 and 2.05 ± 0.02 nmol/mg of microsomal protein, with and without 2,2,2-trifluoroethyl ethyl ether treatment respectively.

Treatment of phenobarbital-induced rats with 2,2,2-trifluoroethyl ethyl ether did not significantly affect the amounts of haem precursors in urine or faeces or the activity of hepatic haem oxygenase. The amounts of the haem precursors per three rats before and after treatment with 2,2,2-trifluoroethyl ethyl ether were as follows: δ-aminolaevulinate, 58.2 ± 12.7 and 78.3 ± 10.0 μg/24 h; porphobilinogen, 5.6 ± 2.0 and 3.9 ± 1.2 μg/24 h; uroporphyrin in urine, 1.8 ± 0.6 and 2.1 ± 0.9 μg/24 h; coproporphyrin in urine, 19.4 ± 1.7 and 19.8 ± 0.8 μg/24 h; coproporphyrin in faeces, 11.2 ± 0.8 and 12.3 ± 2.0 μg/g dry wt.; protoporphyrin in faeces, 44.6 ± 3.9 and 40.9 ± 2.4 μg/g dry wt. The activity of haem oxygenase was 0.04 ± 0.005 nmol of bilirubin/min per mg of protein with or without 2,2,2-trifluoroethyl ethyl ether treatment.

**Effect of chronic fluroxene treatment on the amounts of hepatic microsomal cytochrome P-450 and haem**

Uninduced Wistar rats were repeatedly treated with fluroxene (1 ml/48 h per kg) for a period of 22 days, the last injection of fluroxene being given 16 h before the animals were killed. Control rats were untreated, but housed under similar conditions for 22 days. The concentrations of cytochrome P-450 were 1.08 ± 0.23 and 1.11 ± 0.28 nmol/mg of microsomal protein and of haem were 1.71 ± 0.44 and 1.81 ± 0.17 nmol/mg of microsomal protein with and without chronic fluroxene administration respectively.

**Effect of chronic fluroxene treatment on haem metabolism**

The amounts of δ-aminolaevulinate and porphobilinogen in urine were generally increased after chronic fluroxene treatment (Table 4). Extremely high concentrations of porphobilinogen (elevated about 20-fold) were found in the urine of experimental animals towards the end of both experiments. Amounts of uroporphyrin in urine were initially increased by fluroxene, but subsequently decreased until the amounts were comparable with the control value. Amounts of coproporphyrin in urine were slightly increased after one fluroxene injection and remained elevated throughout the experiment by 2–3-fold (Table 4). The faeces of rats chronically treated with fluroxene showed slightly decreased amounts of porphyrins; coproporphyrin decreased from 4.9 to 2.6 μg/48 h per rat, whereas protoporphyrin decreased from 13.9 to 10.6 μg/48 h per rat over the course of the experiment.

The activity of δ-aminolaevulinate synthase was significantly elevated after ten injections of fluroxene. The activity of δ-aminolaevulinate synthase was 0.042 ± 0.002 and 0.021 ± 0.003 μg of δ-aminolaevulinate formed/30 min per mg of protein with and without fluroxene treatment (P < 0.001).

The activity of hepatic uroporphyrinogen synthase was significantly (P < 0.001) decreased in rats after ten injections of fluroxene. The activity of the
Table 3. **Effect of fluroxene on the amounts of haem precursors in the urine and faeces and on the activity of hepatic δ-aminolaevulinate synthase**

Rats were pretreated with inducing agents and injected with fluroxene as described in the Materials and methods section. Urine and faeces were collected from groups of three identically treated rats over a period of 24h, commencing immediately after the fluroxene injection or at a comparable time for rats not treated with fluroxene. Rats were killed 16h after fluroxene treatment for the determination of δ-aminolaevulinate synthase. Assays are as described in the Materials and methods section. Reported values are means ± S.D. for determinations on each of three to four groups of three rats.

<table>
<thead>
<tr>
<th>Induction of rats</th>
<th>Dosage of fluroxene (mg/kg)</th>
<th>δ-Amino-laevulinate (µg/24h per three rats)</th>
<th>Porphobilinogen (µg/24h per three rats)</th>
<th>Uroporphyrin (µg/24h per three rats)</th>
<th>Coproporphyrin (µg/24h per three rats)</th>
<th>Coproporphyrin (µg/g dry wt. per three rats)</th>
<th>Protoporphyrin (µg/g dry wt. per three rats)</th>
<th>Δ-Aminolaevulinate synthase (µg of δ-aminolaevulinate/30min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
<td>0</td>
<td>91 ± 27</td>
<td>11 ± 4</td>
<td>2.5 ± 0.2</td>
<td>37.6 ± 18.8</td>
<td>11.3 ± 2.8</td>
<td>26.9 ± 7.0</td>
<td>-</td>
</tr>
<tr>
<td>None</td>
<td>4.0</td>
<td>162 ± 60†</td>
<td>9 ± 4</td>
<td>1.6 ± 0.6†</td>
<td>21.3 ± 13.3</td>
<td>13.0 ± 3.8</td>
<td>35.2 ± 12.9</td>
<td>0.024 ± 0.005</td>
</tr>
<tr>
<td>None</td>
<td>4.0</td>
<td>52 ± 24</td>
<td>6 ± 3</td>
<td>1.6 ± 1.6</td>
<td>8.1 ± 4.1</td>
<td>6.2 ± 2.5</td>
<td>35.0 ± 15.8</td>
<td>0.038 ± 0.006§</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>81 ± 29†</td>
<td>14 ± 6†</td>
<td>1.9 ± 2.0</td>
<td>7.1 ± 5.8</td>
<td>8.0 ± 3.0</td>
<td>32.8 ± 14.0</td>
<td>0.023 ± 0.004§</td>
</tr>
<tr>
<td>None</td>
<td>6.0</td>
<td>62 ± 25</td>
<td>8 ± 5</td>
<td>0.8 ± 0.7</td>
<td>13.9 ± 3.2</td>
<td>9.7 ± 5.1</td>
<td>43.8 ± 8.9</td>
<td>0.035 ± 0.010‡‡</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>0</td>
<td>75 ± 27</td>
<td>13 ± 6†</td>
<td>0.8 ± 0.3</td>
<td>8.0 ± 2.0</td>
<td>10.4 ± 3.3</td>
<td>39.8 ± 7.4</td>
<td>0.021 ± 0.004</td>
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<tr>
<td>Phenobarbital</td>
<td>4.0</td>
<td>36 ± 12</td>
<td>4 ± 1</td>
<td>2.2 ± 0.9</td>
<td>25.6 ± 5.8</td>
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<td>80.9 ± 35.0</td>
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<td>Phenobarbital</td>
<td>0</td>
<td>79 ± 20</td>
<td>26 ± 16†</td>
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<tr>
<td>Phenobarbital</td>
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<td>67 ± 22</td>
<td>9 ± 6</td>
<td>1.8 ± 1.5</td>
<td>34.7 ± 11.7</td>
<td>15.9 ± 3.5</td>
<td>49.6 ± 7.1</td>
<td>0.047 ± 0.010‡‡</td>
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</table>

† Probably differs from identically induced rats not treated with fluroxene, *P* < 0.05.
‡ Differs significantly from identically induced rats not treated with fluroxene, *P* < 0.01.
§ Differs significantly from identically induced rats not treated with fluroxene, *P* < 0.001.
|| Obtained by using Student's *t* test for paired data, *P* < 0.01.
Fluroxene and haem metabolism

Table 4. Effect of chronic fluroxene treatment on the amounts of haem precursors in urine

Male Wistar rats were injected with fluroxene (1ml/kg) every 48h. Urine was collected from groups of three rats over a period of 48h, commencing immediately after each injection of fluroxene. The results are averages for two experiments, except for 7–10 fluroxene injections, which represent the results of single experiments. Results are means and given in µg/48h per rat. For each experiment, s.d. values are as follows: δ-aminolaevulinate, 0.6; porphobilinogen, 0.3; uroporphyrin, 0.2; coproporphyrin, 1.5.

<table>
<thead>
<tr>
<th>No. of injections of fluroxene</th>
<th>δ-Aminolaevulinate</th>
<th>Porphobilinogen</th>
<th>Uroporphyrin</th>
<th>Coproporphyrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19.1</td>
<td>1.2</td>
<td>0.5</td>
<td>3.5</td>
</tr>
<tr>
<td>1</td>
<td>35.0†</td>
<td>1.4</td>
<td>1.6</td>
<td>7.5</td>
</tr>
<tr>
<td>2</td>
<td>23.8*</td>
<td>5.5†</td>
<td>1.3</td>
<td>10.5</td>
</tr>
<tr>
<td>3</td>
<td>21.0</td>
<td>3.8*</td>
<td>0.9</td>
<td>7.0</td>
</tr>
<tr>
<td>4</td>
<td>21.8</td>
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<td>6.5</td>
</tr>
<tr>
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<td>5.8†</td>
<td>1.1</td>
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</tr>
<tr>
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<td>10.6‡</td>
<td>0.7</td>
<td>7.6</td>
</tr>
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<td>9.7†</td>
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<td>10.2†</td>
<td>—</td>
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<td>29.8‡</td>
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<td>54.0‡</td>
<td>32.1‡</td>
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</tr>
</tbody>
</table>

* Probably differs from controls, P < 0.05.
† Differs significantly from controls, P < 0.01.
‡ Differs significantly from controls, P < 0.001.

The activity of haem oxygenase was unaffected by chronic fluroxene treatment, with the activity of the enzyme being 0.12 ± 0.04 and 0.11 ± 0.03 nmol of bilirubin formed/10 min per mg of protein with and without fluroxene treatment.

Discussion

Fluroxene is shown to degrade the haem of hepatic microsomal cytochrome P-450 and to affect haem biosynthesis and degradation. Single injections of fluroxene resulted in comparable decreases in the amounts of hepatic microsomal cytochrome P-450 and haem, and the magnitude of these losses was increased by the prior induction of cytochrome P-450 with phenobarbital and 3-methylcholanthrene (Table 1). These effects are similar to those observed after fluroxene anaesthesia (Ivanetich et al., 1976a).

Acute fluroxene treatment was also found to stimulate the endogenous pathways for haem biosynthesis and degradation. Single injections of fluroxene into uninduced rats resulted in increased activity of δ-aminolaevulinate synthase, the rate-limiting enzyme of the haem-biosynthetic pathway, and in increased amounts of δ-aminolaevulinate and porphobilinogen in the urine. Prior treatment with phenobarbital and 3-methylcholanthrene in general enhanced the effects of fluroxene on the haem-biosynthetic pathway (Table 3).
propylacetamide is proposed to affect haem biosynthesis, namely by degrading the haem moiety of cytochrome P-450 by a non-physiological pathway in which cytochrome P-450 may itself provide the catalytic centre for the chemical modification of the haem (Unseld & De Matteis, 1978).

Allylisopropylacetamide and fluroxene do, however, differ in their effects on porphyrins in urine and faeces and on haem oxygenase. Allylisopropylacetamide, but not fluroxene, increases the amounts of porphyrins, whereas fluroxene, but not allylisopropylacetamide, stimulates hepatic haem oxygenase (Tables 1 and 3) (Schmid & Schwartz, 1952; Schmid et al., 1955; Goldberg et al., 1955; Bissell & Hammaker, 1976b; Liem & Muller-Eberhard, 1976). Thus there appears to be a second possible mechanism by which fluroxene, but not allylisopropylacetamide, could deplete the hepatic regulatory haem pool, namely by elevating the activity of hepatic haem oxygenase, which could result in a marked stimulation of the physiological pathway for haem biodegradation. The elevation of hepatic haem oxygenase in fluroxene-treated rats also appears to be linked to the fluroxene-mediated degradation of cytochrome P-450 (Table 2).

The mechanisms by which fluroxene enhances the activity of haem oxygenase is, however, uncertain. The concurrent increases in the activities of hepatic haem oxygenase and hepatic δ-aminolaevulinate synthase that were observed in fluroxene-treated animals were unexpected. It was anticipated that a loss of haem from the regulatory haem pool would arise as a consequence of the degradation of the haem of hepatic cytochrome P-450 by fluroxene, and that this loss would be associated with increased activity of hepatic δ-aminolaevulinate synthase and unchanged or slightly decreased activity of hepatic haem oxygenase (see, e.g., Correia & Burk, 1978).

A loss of haem from hepatic cytochrome P-450 has in several cases been found to be accompanied by an increase in the activity of hepatic haem oxygenase (Bissell & Hammaker, 1976a; Correia & Burk, 1978). However, substances such as adrenaline (epinephrine), endotoxins and carbon disulphide, which exert these effects, appear to do so by decreasing the affinity of apo-(cytochrome P-450) for haem, thereby causing increased amounts of haem to enter the regulatory pool (Bakken et al., 1972; Gemsa et al., 1974; Bissell & Hammaker, 1976a; Järvisalo et al., 1978). This mechanism does not appear to be operative for fluroxene in as much as fluroxene stimulates the chemical modification of the haem of cytochrome P-450 and not the dissociation of haem from this protein (Ivanetich et al., 1975).

Certain heavy metals, e.g. cobalt, are thought to cause induction de novo of hepatic haem oxygenase (Maines & Kappas, 1977). With cobalt treatment, the increase in the activity of hepatic haem oxygenase is followed by a decrease in microsomal cytochrome P-450 and haem in the liver (Maines & Kappas, 1976a). Such a mechanism, where the induction of haem oxygenase in the liver results in an enhanced breakdown of the haem of pre-existing cytochrome P-450 and of the regulatory haem pool is in clear contrast with the sequence of events observed with fluroxene (Table 2).

It is possible that fluroxene itself or the product of the chemically modified haem moiety, presumably a fluroxene—haem or fluroxene—porphyrin adduct (J. J. Bradshaw & K. M. Ivanetich, unpublished work), may act as an inducing agent for hepatic haem oxygenase. Alternatively, the iron ion of the chemically modified haem moiety might exert this effect in as much as iron is known to affect haem metabolism (see, e.g., Maines & Kappas, 1976b).

Although the amounts of hepatic cytochrome P-450 appear to play an important part in determining the rates of haem biosynthesis and biodegradation in the liver, there are other mechanisms whereby these pathways may be affected. For example, some chemicals are known to affect haem biosynthesis by inhibiting one enzyme of the biosynthetic pathway, thus causing a partial block in haem biosynthesis (Tephy et al., 1971; De Matteis & Gibbs, 1976; Stonard, 1978). It is possible that the decreased activity of hepatic uroporphyrinogen synthase seen after chronic fluroxene treatment (see the Results section) may reflect the inhibition of this enzyme by fluroxene or a metabolite thereof.

In the case of animals treated chronically with fluroxene, where the activity of uroporphyrinogen synthase is measurably decreased, the resulting decreased flux through the biosynthetic pathway would be expected to decrease the repression by haem of δ-aminolaevulinate synthase in the liver (Strand et al., 1972; Sassa et al., 1974; Doss & Tiepermann, 1978). Inasmuch as the amounts of hepatic microsomal cytochrome P-450 and haem were not decreased after chronic treatment of animals with fluroxene (see the Results section), it is not known whether the stimulation of haem biosynthesis by chronic exposure to fluroxene (Table 4) was a consequence of the degradation of the haem of cytochrome P-450 in the liver by fluroxene (which could have been evident at an earlier time) or of an inhibition of uroporphyrinogen synthase by fluroxene.

The human genetic diseases known as the hepatic porphyrias, which are characterized by excessive quantities of porphyrin precursors and/or porphyrins in the urine or faeces, are often a result of a genetically determined defect in one of the enzymes of the haem-biosynthetic pathway (Elder et al., 1972; Brodie et al., 1977; Walsh, 1977; Stonard, 1980).
1978). Acute intermittent porphyria, which is one of several types of human genetic porphyria, is characterized biochemically by a defect in uroporphyrinogen synthase, such that the activity of this enzyme is decreased to approx. 50% of the normal value (Strand et al., 1972; Sassa et al., 1974; Romeo, 1977; Anderson et al., 1979). The decreased activity of uroporphyrinogen synthase results in secondary de-repression of hepatic δ-aminolaevulinate synthase and in the overproduction and excretion of the porphyrin precursors, δ-aminolaevulinate and porphobilinogen (Strand et al., 1972). Inasmuch as prolonged administration of fluroxene produces a biochemical picture that resembles acute intermittent porphyria, it is possible that chronic treatment of laboratory rodents with fluroxene might be used as a model for the study of this disease.

It has recently been established that a wide variety of compounds, including nitro- and halo-alkanes, nitriles and several compounds that contain unsaturated carbon–carbon bonds, are capable of chemically modifying the haem of hepatic microsomal cytochrome P-450 (Ivanetich et al., 1977; Guengrich & Strickland, 1977; Ivanetich et al., 1978; White, 1978). Inasmuch as these compounds appear to modify the haem of hepatic microsomal cytochrome P-450, it might be anticipated that these compounds could also affect haem biosynthesis and degradation.

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