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REACTION SCHEMES FOR THE DEGRADATION OF CYTOCHROMES P-450 BY ALLYL-ISO-PROPYLACETAMIDE AND FLUROXENE*

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Abstract—The degradation of the heme of hepatic microsomal cytochromes P-450 by fluroxene (2,2,2-trifluoroethyl vinyl ether) and allyl-iso-propylacetamide (AIA) was characterized. K_M and V_{max} values and pseudo first-order rate constants for the degradation of the heme of hepatic microsomal cytochromes P-450 by AIA and pseudo first-order rate constants for the metabolism of fluroxene to 2,2,2-trifluoroethanol were measured. Based on these results and on data from the literature, eight possible reaction schemes were proposed for the degradation of cytochromes P-450 by AIA and fluroxene. The reaction schemes were evaluated by computer analysis in terms of how closely they could mimic the experimental results for the degradation of cytochromes P-450 *in vitro* and *in vivo*. It was concluded that suitable reaction schemes for the degradation of the heme of hepatic microsomal cytochromes P-450 by AIA and fluroxene incorporated the following characteristics: Transient reactive species of AIA and fluroxene were formed by at least two forms of cytochrome P-450. The reactive species, once produced, degraded the same form of the enzyme (e.g. phenobarbital-induced cytochrome P-450) and possibly even the same enzyme molecule that produced it. It appears that the enzyme–substrate complex that gives rise to the production of the transient reactive species may be distinct from the typical cytochrome P-450–substrate complex that gives rise to a Type I difference spectrum.

The porphyrinogenic compound allyl-iso-propylacetamide (AIA) and the anesthetic agent fluroxene (2,2,2-trifluoroethyl vinyl ether) degrade the heme of hepatic cytochromes P-450 *in vivo* and *in vitro* [1–7]. The product of these reactions appears to be an adduct of a metabolically activated form of either compound with a vinyl side chain of the heme moiety of cytochromes P-450 [8] (K. M. Ivanetich and J. J. Bradshaw, unpublished results). The enzyme system catalyzing the metabolic activation of these compounds appears to be the hepatic microsomal cytochrome P-450 enzyme system, but there does not appear to be a clear relation between the extent of metabolism of each compound by the cytochrome P-450 system and the extent of its degradation of cytochromes P-450 [1, 2, 5, 7].

AIA does not produce a difference spectrum with hepatic microsomal cytochromes P-450 and appears not to be extensively metabolized to stable, readily isolable products by this enzyme system, yet the extent of degradation of cytochromes P-450 by AIA is enhanced by the induction of cytochrome P-450 with phenobarbital [1, 3, 5, 9].

In contrast to AIA, fluroxene binds as a Type I substrate to cytochrome P-450, and the first step in the metabolism of fluroxene *in vivo* and *in vitro* (i.e. its conversion to 2,2,2-trifluoroethanol and other products) is known to be catalyzed by the hepatic microsomal cytochrome P-450 system [7, 10]. A form of cytochrome P-450 that is induced by phenobarbital appears to be the major form of the enzyme that binds and metabolizes fluroxene. Cytochrome P-448, the form of cytochrome P-450 induced by 3-methylcholanthrene, does not appear to bind fluroxene or convert it to 2,2,2-trifluoroethanol to a measurable extent [7, 10]. The degradation of cytochromes P-450 by fluroxene is, however, stimulated both by the induction of cytochrome P-450 with phenobarbital and by the induction of cytochrome P-448 with 3-methylcholanthrene [5–7, 10].

The specificities of AIA and fluroxene in degrading different forms of cytochrome P-450 differ: AIA is nonspecific for several types of cytochrome P-450 in phenobarbital-induced rats, but appears to specifically degrade the form of the enzyme induced by phenobarbital in rats pretreated with 3-methylcholanthrene. Fluroxene also nonspecifically degrades multiple forms of cytochrome P-450 in phenobarbital-induced rats but, in contrast to AIA, preferentially degrades cytochrome P-448 in 3-methylcholanthrene-induced rats [11].

We have attempted to gain further insight into the mechanisms of the degradation of hepatic cytochromes P-450 by AIA and fluroxene. Toward this aim, the degradation of cytochromes P-450 by these compounds and the production of 2,2,2-trifluoroethanol from fluroxene have been characterized experimentally by determining—where not available in the literature—the pseudo first-order rate con-

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stants (k_{obs}), K_M and V_{max} values, and other parameters characteristic of these reactions. Based on the results reported here and elsewhere, several possible reaction schemes have been proposed. These schemes were evaluated, using computer analysis, in terms of how closely they were able to mimic the experimental results for the degradation of cytochrome P-450 by AIA and fluroxene and for the metabolism of fluroxene to 2,2,2-trifluoroethanol by cytochrome P-450.

Specifically, the reaction schemes were chosen to assess: (1) which forms of cytochrome P-450 convert AIA and fluroxene to reactive metabolities that are capable of chemically modifying the heme of cytochromes P-450; (2) whether AIA and fluroxene give rise to reactive metabolites that are transient enzyme-bound species; and (3) whether reactive species produced from AIA or fluroxene by one form of cytochrome P-450 can degrade another form of the enzyme.

EXPERIMENTAL

Materials. Fluroxene (2,2,2-trifluoroethyl vinyl ether) was purchased from Ohio Medical Products, Madison, WI, U.S.A. Ethyl vinyl ether and divinyl ether were from Fluka, Buchs, Switzerland, and Maybaker, Port Elizabeth, South Africa, respectively. 2,2,2-Trifluoroethanol and trifluoroacetic acid were purchased from Merck Chemicals, Darmstadt, F.R.G. Trifluoroacetaldehyde was from ICN Pharmaceuticals, Plainview, NY, U.S.A. 2,2,2-Trifluoroethyl ethyl ether was prepared as described previously [5]. AIA was a gift from Hoffman–La Roche Ltd., Nutley, NJ, U.S.A. NADPH and the components of the NADPH-generating system were obtained from Miles Laboratories, Cape Town, South Africa. EDTA was obtained from BDH Chemicals, Poole, U.K. Cylinders of compressed gases were from Afrox Ltd., Cape Town, South Africa. Water was distilled and deionized.

Treatment of animals. Male Wistar rats (180–220 g) were used for all experiments. Animals were given phenobarbital or 3-methylcholanthrene by intraperitoneal injection as described earlier [6].

Preparation of hepatic microsomes. Hepatic microsomes were prepared by gel filtration on Sepharose 2B equilibrated with 0.15 M KCl–0.02 M Tris–HCl, pH 7.4, according to the method of Tangen *et al.* [12]. The microsomes were used in all experiments at a concentration of 2 mg protein/ml 0.02 M Tris–HCl, pH 7.4. Protein concentration was determined by the method of Lowry *et al.* [13], as modified by Chaykin [14], using bovine serum albumin as standard.

Hepatic microsomal NADPH oxidation. The oxidation of NADPH was monitored spectrally at 340 nm at 30° [10]. To the sample cuvette containing 3 ml hepatic microsomes was added fluroxene or 2,2,2-trifluoroethyl ethyl ether, and the mixture was vortex mixed for 30 sec. Where required, AIA was then added, and the reaction was initiated with the addition of NADPH (0.2 mM, final concentration). Reference cuvettes contained microsomal suspension only. Background rates of NADPH oxidation were measured in the presence of substrate under an atmosphere of $CO:O_2$ (80:20 v/v) [15].

Composition of incubation mixtures. For the determination of K_M and V_{max} values for the degradation of cytochrome P-450 by AIA, incubation mixtures contained hepatic microsomes (2 mg protein/ml), EDTA (0.2 mM) and an NADPH-generating system (0.4 mM NADP, 7.5 mM glucose-6-phosphate, 0.5 units/ml glucose-6-phosphate dehydrogenase, 1 mM nicotinamide and 5 mM $MgCl_2$) and variable amounts of AIA in 0.02 M Tris–HCl, pH 7.4, the reaction being initiated with the addition of the NADPH-generating system–EDTA mixture. The losses of cytochrome P-450 reported as a function of time are relative to zero time samples of identical composition. The reported values have been corrected for the losses of cytochrome P-450 seen with incubation mixtures prepared as above, but in the absence of AIA. Incubations were at 30° with shaking at 60 cycles/min for 7 min. References for the spectral assay comprised hepatic microsomal suspensions that had been incubated for the same period of time as the samples.

For studies of the degradation of hepatic microsomal cytochromes P-450 by analogues and metabolites of fluroxene, incubations were carried out as described for AIA, except that AIA was omitted from the reaction mixture and the analogues and metabolites were, where necessary, dispersed in the microsomal suspension by vortex mixing for 30 sec. An incubation time of 30 min was used in these experiments.

For determination of the first-order rate constants for the degradation of cytochromes P-450 by AIA and for the metabolism of fluroxene to 2,2,2-trifluoroethanol, incubation mixtures were prepared as described above, except that 5.0 mM AIA or 30 mM fluroxene was added to the hepatic microsomes before the initiation of the reaction, and the fluroxene was dispersed in the microsomal suspension by vortex mixing for 30 sec. At every eighth minute additional 0.4 mM NADP, 7.5 mM glucose-6-phosphate and 0.5 units/ml glucose-6-phosphate dehydrogenase were added to the incubation mixture through the serum cap. A separate incubation mixture was used for each time point required. Incubations were at 30° with shaking at 60 cycles/min for 0–65 min.

Assays. The levels of hepatic microsomal cytochromes P-450 were determined from the difference spectrum of CO-ferrocytochrome P-450 versus ferrocytochrome P-450 according to the method of Omura and Sato [16]. An extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in absorbance between 450 and 490 nm was used [16]. Microsomal heme was determined spectrally as the reduced pyridine hemochrome according to Omura and Sato [16] ($\varepsilon_{557-575\,nm}$ 32.4 mM^{-1} cm^{-1}).

2,2,2-Trifluoroethanol was measured in incubation mixtures by gas–liquid chromatography as described by Gion *et al.* [17] using a Beckman GC-M gas chromatograph. Peak areas were determined by a Pye Unicam DP88 computing integrator.

For all spectral studies, a Pye Unicam SP1800 spectrophotometer was used. The thermostatically controlled compartment adjacent to the photomultiplier designed to accommodate turbid samples was used for all microsomal samples.

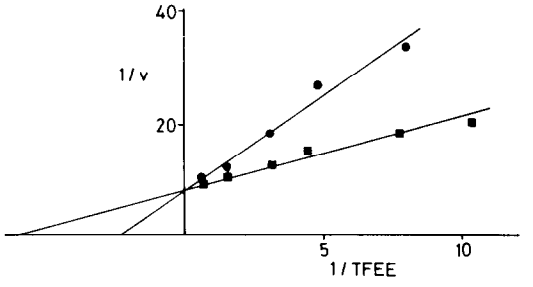

Fig. 1. Lineweaver–Burk plot of the inhibition by AIA of the stimulation of hepatic microsomal NADPH oxidation by 2,2,2-trifluoroethyl ethyl ether (TFEE). Key: v, $A_{340\,nm}$/min; TFEE concentration, mM. Reaction mixtures contained hepatic microsomes from phenobarbital-induced rats (2.0 mg protein/ml), NADPH (0.2 mM), and 2,2,2-trifluoroethyl ethyl ether in the presence (●) and absence (■) of 3 mM AIA. Reaction temperature was 30°.

Calculations and statistical analysis. Inhibition constants (K_i), Michaelis (K_M) constants and maximal rates of metabolism (V_{max}) were calculated from Hanes and Eadie Hofstee plots. Pseudo first-order rate constants (k_{obs}) were calculated from plots of $\ln(A_\infty - A_t)$ vs time where A_∞ and A_t are the absorbances (or concentrations) of the substance at infinity and at time t, respectively.*

Student's t-test for unpaired data was used to assess the significance of the difference between means. The difference was considered significant when $P < 0.01$. Results are reported as means ± S.D.

Reaction schemes were assessed using the mimic program on a Univac model 1180 computer. The mimic program—a digital simulation program—was written by H. E. Petersen and F. J. Samson of the Systems Engineering Group of Wright–Patterson Air Force Base in May 1965. The program was updated in July 1967 by the I.S.D. systems group of Univac and is commercially available from this firm.

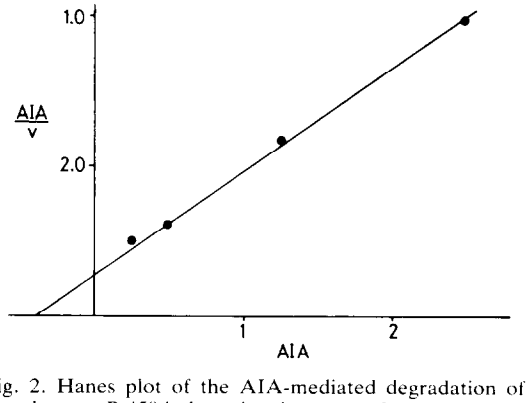

Fig. 2. Hanes plot of the AIA-mediated degradation of cytochromes P-450 in hepatic microsomes from phenobarbital-induced rats. Key: AIA concentration, mM; v, rate in μM cytochromes P-450/7 min. Reaction mixtures contained hepatic microsomes (2 mg protein/ml), EDTA (0.2 mM), NADPH-generating system, and AIA in 0.02 M Tris–HCl, pH 7.4. Incubations were at 30° for 7 min.

* Relative to the concentration of cytochromes P-450 (*ca.* 3 μM).

RESULTS AND DISCUSSION

Interaction of AIA with the hepatic microsomal cytochrome P-450 system. Because AIA does not reproducibly give rise to a difference spectrum of significant magnitude with hepatic microsomal cytochromes P-450 [8], the equilibrium constants for the interaction of AIA with cytochromes P-450 were calculated from measurements of the inhibition by AIA of the hepatic microsomal metabolism of fluroxene and 2,2,2-trifluoroethyl ethyl ether. The metabolism of these anesthetic ethers was assessed by their stimulation of CO-inhibitable NADPH oxidation, which has been shown to be equivalent to the production of 2,2,2-trifluoroethanol from these compounds [7, 10].

With microsomes from phenobarbital-induced rats, AIA competitively inhibited the fluroxene and 2,2,2-trifluoroethyl ethyl ether mediated stimulation of CO-inhibitable NADPH oxidation (see, e.g., Fig. 1). The K_i values for AIA were 1.19 mM and 1.04 mM in the presence of fluroxene and 2,2,2-trifluoroethyl ethyl ether, respectively. Inasmuch as these K_i values are similar to the K_i of 1.03 mM calculated from the data of Sweeney and Rothwell [9] for the inhibition by AIA of the binding of hexobarbital to cytochromes P-450 in hepatic microsomes from phenobarbital-induced rats, it would appear that NADPH had no effect on the K_i for AIA.

K_M and V_{max} *for the degradation of cytochromes P-450 by AIA.* The rate of degradation of cytochromes P-450 by AIA in hepatic microsomes from phenobarbital-induced rats was linear for approximately 7 min. The K_M and V_{max} values for the degradation of hepatic microsomal cytochromes P-450 by AIA under these conditions were, therefore, calculated using the initial rate of degradation of cytochromes P-450 over this time period.

The degradation of cytochromes P-450 by AIA in phenobarbital-induced hepatic microsomes was characterized by a K_M of 0.44 ± 0.18 mM and a V_{max} of 0.98 ± 0.16 μM cytochromes P-450 degraded/7 min (Fig. 2). The degradation by AIA of cytochromes P-450 in microsomes from uninduced and 3-methylcholanthrene-induced rats was too slight to permit accurate determination of K_M and V_{max} values.

Kinetics of the degradation of cytochromes P-450 by AIA. The rate of degradation of hepatic microsomal cytochromes P-450 was monitored at an excess concentration* of AIA (5 mM), using microsomes from 3-methylcholanthrene-induced, phenobarbital-induced, and uninduced rats. The degradation of cytochromes P-450 by AIA was pseudo first-order with respect to cytochromes P-450 in microsomes from phenobarbital-induced rats (Fig. 3), with a pseudo first-order rate constant (k_{obs}) of 5.2 ± 0.8 × 10^{-2} min^{-1}. For AIA with microsomes from 3-methylcholanthrene-induced or untreated rats, the degradation of cytochromes P-450 was too slight to permit the determination of the order of the reaction or the calculation of rate constants. With microsomes from untreated, 3-methylcholanthrene-induced and phenobarbital-induced rats, the degradation of cytochromes P-450 by AIA was 0.30 ± 0.02, 0.46 ± 0.10 and 2.58 ± 0.24 μM/65 min.

Kinetics of the production of 2,2,2-trifluoroethanol

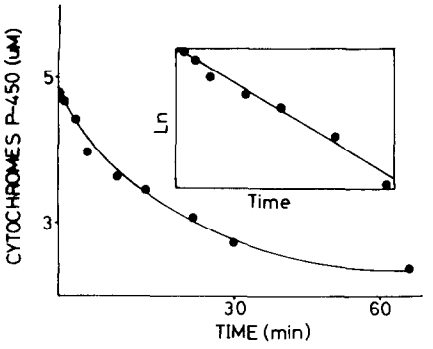

Fig. 3. Degradation of hepatic microsomal cytochromes P-450 by AIA as a function of time in microsomes from phenobarbital-induced rats. Inset: ln[cytochromes P-450] vs time. Reaction mixtures contained hepatic microsomes (2 mg protein/ml), EDTA (0.2 mM), NADPH-generating system, and AIA (5 mM). Incubations were at 30°.

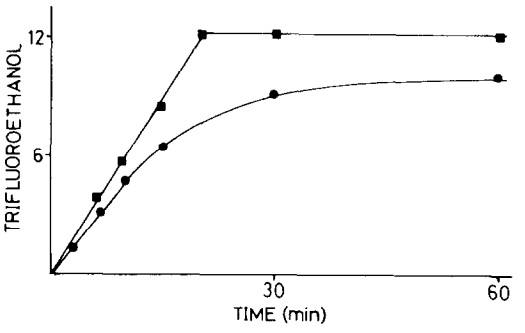

Fig. 4. Production of 2,2,2-trifluoroethanol from fluroxene (●) and from 2,2,2-trifluoroethyl ethyl ether (■) as a function of time by hepatic microsomes from phenobarbital-induced rats. Trifluoroethanol concentration, arbitrary units. Incubation mixtures contained hepatic microsomes (2 mg protein/ml), EDTA (0.2 mM), NADPH-generating system, and fluroxene (30 mM) or 2,2,2-trifluoroethyl ethyl ether (30 mM) in 0.02 M Tris–HCl, pH 7.4, at 30°.

from fluroxene and 2,2,2-trifluoroethyl ethyl ether. The production of 2,2,2-trifluoroethanol from fluroxene followed pseudo first-order kinetics in microsomes from phenobarbital- and 3-methylcholanthrene-treated or untreated rats (see, e.g., Fig. 4). The first-order rate constants for the production of 2,2,2-trifluoroethanol from fluroxene for microsomes from untreated, 3-methylcholanthrene- and phenobarbital-pretreated rats were found to be $4.3 \pm 1.0 \times 10^{-2}$, $3.4 \pm 0.9 \times 10^{-2}$ and $3.7 \pm 1.0 \times 10^{-2}$ min^{-1}. The reaction was expected to be pseudo first-order as a consequence of the pseudo first-order degradation of cytochromes P-450. The production of 2,2,2-trifluoroethanol was 250 ± 10, 236 ± 10 and 728 ± 30 μM/65 min for microsomes from untreated, 3-methylcholanthrene-induced and phenobarbital-induced rats, respectively.

The production of 2,2,2-trifluoroethanol from 2,2,2-trifluoroethyl ethyl ether—which does not degrade cytochromes P-450 [5, 6]—followed zero-order kinetics with microsomes from phenobarbital-induced rats (Fig. 4). A rate of 33 ± 3 μM 2,2,2-trifluoroethanol/min was obtained for microsomes from phenobarbital-induced rats.

Effects of metabolites and analogues of fluroxene on the levels of hepatic microsomal cytochromes P-450 and heme in vitro. None of the reported or proposed metabolites of fluroxene, including 2,2,2-trifluoroethanol (69 mM), trifluoroacetaldehyde (37 mM), trifluoroacetate (200 mM) or bicarbonate (32 mM)* [7, 18, 19], had any effect on the level of hepatic microsomal cytochromes P-450 or heme in microsomes from 3-methylcholanthrene-induced rats, under conditions where fluroxene degraded considerable amounts of this hemoprotein (data not shown). These results are identical to those previously obtained with phenobarbital-induced rats [20]†.

The analogues of fluroxene—ethyl vinyl ether and divinyl ether—significantly decreased the levels of cytochromes P-450 and heme in hepatic microsomes from 3-methylcholanthrene-induced rats, the losses of heme/cytochromes P-450 being 0.21 ± 0.13/0.22 ± 0.03 and 0.38 ± 0.04/0.55 ± 0.04 for ethyl vinyl ether and divinyl ether, respectively. 2,2,2-Trifluoroethyl ethyl ether was without effect on the level of cytochromes P-450 in hepatic microsomes from 3-methylcholanthrene-induced rats (data not shown). These results demonstrate that the heme of cytochromes P-450 is not modified by any known stable metabolite of fluroxene in microsomes from 3-methylcholanthrene-induced rats and suggest that the degradation of cytochromes P-450 is mediated by an activated form of the vinyl moiety under these conditions.

Reaction schemes for the degradation of cytochromes P-450 by AIA and fluroxene. Six reaction schemes, chosen on the basis of experimental data reported here and elsewhere, were devised for the degradation of hepatic microsomal cytochrome P-450 hemoproteins by AIA and fluroxene. The proposed reaction schemes for fluroxene are presented in Table 1. Because the cytochrome P-450-dependent conversion of fluroxene to 2,2,2-trifluoroethanol has been well characterized, this reaction is included in the schemes for fluroxene. The reaction schemes for AIA are identical to those for fluroxene except that k_3 (the rate constant for the production of 2,2,2-trifluoroethanol) has been set to zero in the schemes for AIA since the metabolism of AIA by cytochromes P-450 occurs, at most, only at a slow rate and has not been well characterized.

For the reaction schemes, the levels of the substrates and the various forms of cytochrome P-450 were as follows: The levels of AIA and fluroxene

* The only known metabolite not assayed was 2,2,2-trifluoroethanol glucuronide, a derivative that is thought to be devoid of physiological effects.

† Bicarbonate does not affect the level of cytochromes P-450 in microsomes from phenobarbital-induced rats. A preliminary report to the contrary incorrectly used values that were not corrected for the losses of cytochromes P-450 seen in the presence of hepatic microsomes plus an NADPH-generating system [20].

Table 1. Reaction schemes for the degradation of cytochrome P-450 hemoproteins by fluroxene*

Scheme 1†

$$F + PB450 \underset{k_2}{\overset{k_1}{\rightleftarrows}} [F\text{-}PB450] \xrightarrow{k_3} TFE + PB450$$

$$[F\text{-}PB450] \xrightarrow{k_4} M$$

$$M + PB450 \xrightarrow{k_5} X450$$

$$M + P448 \xrightarrow{k_6} X448$$

Scheme 2†

$$F + PB450 \underset{k_2}{\overset{k_1}{\rightleftarrows}} [F\text{-}PB450] \xrightarrow{k_3} TFE + PB450$$

$$F + P448 \underset{k_6}{\overset{k_5}{\rightleftarrows}} [F\text{-}P448] \xrightarrow{k_7} M + P448 \xrightarrow{k_8} X448$$

$$M + PB450 \xrightarrow{k_4} X450$$

Scheme 3

$$F + PB450 \underset{k_2}{\overset{k_1}{\rightleftarrows}} [F\text{-}PB450] \xrightarrow{k_3} TFE + PB450$$

$$[F\text{-}PB450] \xrightarrow{k_4} X450$$

$$F + P448 \underset{k_6}{\overset{k_5}{\rightleftarrows}} [F\text{-}P448] \xrightarrow{k_7} X448$$

Scheme 4

$$F + PB450 \underset{k_2}{\overset{k_1}{\rightleftarrows}} [F\text{-}PB450] \xrightarrow{k_3} TFE + PB450$$

$$[F\text{-}PB450] \underset{k_5}{\overset{k_4}{\rightleftarrows}} [F\text{-}PB450]\ddagger \xrightarrow{k_6} X450$$

$$F + P448 \underset{k_8}{\overset{k_7}{\rightleftarrows}} [F\text{-}P448] \xrightarrow{k_9} X448$$

Scheme 5

$$F + PB450 \underset{k_2}{\overset{k_1}{\rightleftarrows}} [F\text{-}PB450] \xrightarrow{k_3} TFE + PB450$$

$$F + PB450 \underset{k_5}{\overset{k_4}{\rightleftarrows}} [F\text{-}PB450]\ddagger \xrightarrow{k_6} X450$$

$$F + P448 \underset{k_8}{\overset{k_7}{\rightleftarrows}} [F\text{-}P448] \xrightarrow{k_9} X448$$

Scheme 6

$$F + PB450 \underset{k_2}{\overset{k_1}{\rightleftarrows}} [F\text{-}PB450] \xrightarrow{k_3} TFE + PB450$$

$$F + PB450 \xrightarrow{k_4} X450$$

$$F + P448 \xrightarrow{k_5} X448$$

* Abbreviations used are: F, fluroxene; PB450, phenobarbital-induced form of cytochrome P-450; P448, cytochrome P-448; X450 and X448, degraded forms of PB450 and P448, respectively; TFE, 2,2,2-trifluoroethanol; M, reactive metabolite; and [F-PB450]‡, an enzyme–substrate complex differing from [F-PB450].

† Schemes 1A and 2A are modifications of schemes 1 and 2 where M reacts with the [F-PB450] and [F-PB448] complexes, rather than with free PB450 and P448, in the step of the reaction producing X450 and X448.

were 5.0 mM and 30.0 mM, respectively. The levels of the phenobarbital-inducible form of cytochrome P-450 were set at 1.96 and 0.75 μM in hepatic microsomes from phenobarbital-induced and 3-methylcholanthrene-induced rats, respectively. The levels of cytochrome P-448 were set at 1.4 and 2.0 μM in hepatic microsomes from phenobarbital-induced and 3-methylcholanthrene-induced rats. These levels of cytochrome P-450 hemoproteins were approximations based on information available in the literature [21–27].

The proposed reaction schemes were assessed by comparison with the following experimental data: (1) the K_i values for AIA and the K_s values for fluroxene for their interactions with the hepatic microsomal cytochromes P-450 *in vitro* (see Results and Discussion) [9, 10]; (2) the K_M and V_{max} values for the degradation of cytochromes P-450 by AIA and fluroxene and for the conversion of fluroxene to 2,2,2-trifluoroethanol by hepatic microsomes *in vitro* (see Results and Discussion) [7]; (3) the relative extents of degradation of different forms of cytochrome P-450 by AIA and fluroxene *in vivo* [11]; and (4) the pseudo first-order rate constants (k_{obs}) for the degradation of cytochromes P-450 by AIA and fluroxene and for the production of 2,2,2-trifluoroethanol from fluroxene by hepatic microsomes *in vitro* (see Results and Discussion) [20].

The exact values of the parameters that were used to assess the reaction schemes are given Tables 2–4 for comparative purposes.

In assessing each reaction scheme, the rate constants (e.g. k_1, k_2, k_3, etc.) were adjusted to provide the best correlation between the experimentally determined parameters and the values of the parameters calculated from the model using the mimic program (see Experimental section). Initially, the ratios of k_2/k_1 in all schemes, k_6/k_5 in schemes 2, 2A and 3, k_8/k_7 in schemes 4 and 5, and k_5/k_4 in scheme 5 were set equal to the K_i or K_s value for AIA or fluroxene. For both of these compounds, the absolute values of k_1 and k_2 in each scheme were set greater than the absolute values of k_5 and k_6 in schemes 2, 2A and 3, than k_7 and k_8 in schemes 4 and 5, and than k_4 and k_5 in scheme 5.

The values of the remaining rate constants were adjusted as necessary so that the V_{max} values for the degradation of cytochromes P-450, the V_{max} values for the production of 2,2,2-trifluorethanol, and the ratio of the extents of degradation of cytochrome P-448 and of cytochrome P-450 were equivalent to the experimentally determined values. An error of approximately 10 per cent was considered acceptable for these parameters, and in all cases the correlation of the experimental and calculated results was within this limit (Tables 2–4).

The rate constants of the reaction schemes were also adjusted so that the K_M values for the degradation of cytochromes P-450 and for the production of 2,2,2-trifluoroethanol were as close to the experimental values as possible, but good agreement between the experimental and calculated results was not achievable in all cases (Tables 2–4). The parameters for which the rate constants in the schemes were not adjusted and which were assessed only in the final versions of each scheme were the pseudo

Table 2. Parameters calculated from the final runs of the reaction schemes and the experimentally determined parameters for the degradation by AIA of cytochromes P-450 in hepatic microsomes from phenobarbital-induced rats*

Scheme	K_s (mM)	$\left[\frac{X448/P448}{X450/PB450}\right]$	V_{max}(XTP450) (μM/7 min)	K_M (XTP450) (mM)	$10^2\ k_{obs}$(XTP450) (min^{-1})	XTP450 (μM/65 min)
1	1.0	0.9	1.1	1.0	NC†	3.4
1A	1.0	0.9	1.0	1.0	NC†	3.4
2	1.0	0.9	1.0	1.0	NC†	3.4
2A	1.0	0.9	1.0		NC†	3.4
3	1.0	0.9	1.0	1.0	5.2	3.2
4	1.0	1.0	0.9	0.5	4.9	3.1
5	1.0	0.9	1.1	1.0	5.0	3.2
6	1.0	0.9	1.0	NC	4.8	3.2
	K_i (mM)					
Experimental results‡	1.0	0.9§	1.0	0.44	5.2	2.8

* Abbreviations used are: PB, phenobarbital; TP450, total type P-450 cytochromes; XTP450, degraded or degradation of TP450; PB450, phenobarbital-induced form of cytochrome P-450; X450, degraded PB450; P448, cytochrome P-448; X448, degraded cytochrome P-448; and NC, not calculable.

† Reaction did not follow first-order kinetics.

‡ From this manuscript unless otherwise indicated.

§ Calculated from Ref. 11.

first-order rate constants and the extents of reaction over 65 min for the degradation of cytochromes P-450 and the production of 2,2,2-trifluoroethanol.

For each model, the rate constants that provided the parameters in closest agreement with the experimental results are given in Tables 5 and 6 for AIA and fluroxene respectively. The parameters that were calculated from the different schemes, and the experimentally determined parameters are given in Tables 2–4.

Reaction schemes 1, 1A, 2 and 2A did not follow first-order kinetics for the degradation of cytochromes P-450 by AIA or fluroxene (Tables 2–4). In all cases, plots of the concentration of cytochromes P-450 vs time were sigmoid, with plots of ln[cytochromes P-450] vs time being curved (see, e.g., Fig. 5). In addition, schemes 1 and 1A could not provide differing K_M values for the degradation of cytochromes P-450 by fluroxene and for the production of 2,2,2-trifluoroethanol, as was observed experimentally for microsomes from 3-methylcholanthrene-induced rats (Table 4).

Furthermore, reaction schemes 1, 1A, 2 and 2A did not provide pseudo first-order kinetics for the metabolism of fluroxene to 2,2,2-trifluoroethanol, although in some cases, particularly for 3-methylcholanthrene induction, plots of ln[$TFE_\infty - TFE_t$] were only slightly curved.

Reaction schemes 3–6 followed pseudo first-order kinetics for the degradation of hepatic microsomal cytochromes P-450 by AIA and fluroxene and for the production of 2,2,2-trifluoroethanol. For AIA and fluroxene, all of these schemes except scheme 6—for which K_M values were not calculable—could in large part closely mimic the experimental results. All of these schemes could provide differing K_M values for the degradation of cytochrome P-450 by fluroxene and for the production of 2,2,2-trifluoroethanol from fluroxene (Table 4). For AIA, only scheme 4 was able to provide a K_M value less than K_i, as was observed experimentally, but schemes 3 and 5 provided K_M values that were within a factor of two of the experimentally determined values.

For schemes 3–6, the values of k_{obs} for the degradation of cytochromes P-450 by AIA and fluroxene and for the production of 2,2,2-trifluoroethanol, as well as the extent of production of 2,2,2-trifluoroethanol per 65 min, were similar to the experimental values. All of these schemes, however, for both AIA and fluroxene, gave rise to total extents of degradation of cytochromes P-450 that were equivalent to the total amounts of hepatic microsomal cytochromes P-450, a phenomenon that was not observed experimentally (Tables 2–4). One possible explanation for this discrepancy is that one or more forms of cytochrome P-450, present in hepatic microsomes but not considered in the model reaction schemes.

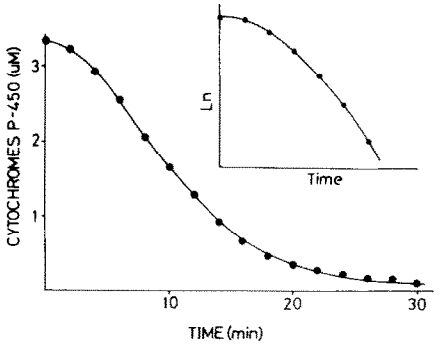

Fig. 5. Degradation of cytochromes P-450 by fluroxene. Data were calculated from reaction scheme 2 for the degradation of cytochromes P-450 in hepatic microsomes from phenobarbital-induced rats by fluroxene (30 mM). Inset: ln[cytochromes P-450] vs time.

Table 3. Parameters calculated from the final runs of the reaction schemes and the experimentally determined parameters for the degradation of cytochromes P-450 by fluroxene and for the metabolism of fluroxene to 2,2,2-trifluoroethanol with microsomes from phenobarbital-induced rats*

Scheme	K_s (mM)	$\left[\frac{\text{X448/P448}}{\text{X450/PB450}}\right]$	V_{max} (μM XTP450/ 7 min)	K_M (X450) (mM)	$10^2\ k_{obs}$ (XTP450) (min^{-1})	XTP450 (μM/65 min)	V_{max} (μM TFE/min)	K_M(TFE) (mM)	$10^2\ k_{obs}$ (TFE) (min^{-1})	TFE (μM/65 min)
1	0.8	0.9	1.0	1.0	NC†	3.4	29	1.0	NC†	347
1A	0.8	1.0	0.9	1.0	NC†	3.4	28	1.0	NC (14.4)†	349
2	0.8	1.0	1.0	0.9	NC†	3.4	29	0.9	NC†	353
2A	0.8	1.0	0.9		NC†	3.4	28	0.9	NC†	343
3	0.8	1.1	1.0	1.1	5.0	3.2	28	1.1	5.5	630
4	0.8	1.0	1.0	0.5	5.3	3.2	28	0.5	5.5	568
5	0.8	1.0	0.9	1.0	4.4	3.1	29	1.0	5.0	689
6	0.8	1.1	0.9	NC	4.2	3.1	29	0.9	4.7	742
Experimental results	0.8‡	1.0§	0.9‖	0.8‖¶	4.3	2.1	28‖	0.7‖	3.7	728

* Abbreviations used are: TFE, 2,2,2-trifluoroethanol; PB, phenobarbital; TP450, total type P-450 cytochromes; XTP450, degraded or degradation of TP450; PB450, phenobarbital-induced form of cytochrome P-450; X450, degraded PB450; P448, cytochrome P-448; X448, degraded cytochrome P-448; and NC, not calculable.

† Reaction did not follow first-order kinetics. Where a numerical value is given in parentheses, the plot of $\ln(TFE_\infty - TFE_t)$ vs time was slightly curved and the value in parentheses represents an apparent k_{obs}, since the reaction was not strictly first order.

‡ From Ref. 10.

§ From Ref. 11.

‖ From Ref. 7.

¶ For XTP450.

Table 4. Parameters calculated from the final runs of the reaction schemes and the experimentally determined parameters for the degradation of cytochromes P-450 by fluroxene and for the metabolism of fluroxene to 2,2,2-trifluoroethanol with microsomes from 3-methylcholanthrene-induced rats*

Scheme	K_s (mM)	$\left[\frac{\text{X448/P448}}{\text{X450/PB450}}\right]$	V_{max} (μM XTP450/ 7 min)	K_M (X448) (mM)	$10^2\ k_{obs}$ (XTP450) (min^{-1})	XTP450 (μM/65 min)	V_{max} (μM TFE/min)	K_M(TFE) (mM)	$10^2\ k_{obs}$ (TFE) (min^{-1})	TFE (μM/65 min)
1	0.8	2.9	1.0	1.3	NC†	2.7	9.5	1.3	NC (7.6)†	174
1A	0.8	2.8	1.0	1.2	NC†	2.7	9.5	1.2	NC (8.1)†	166
2	0.8	2.8	1.0	3.3	NC†	2.7	9.5	1.3	NC (6.7)†	195
2A	0.8	2.8	1.0		NC†	2.7	9.5	NC	NC (6.3)†	197
3	0.8	2.8	1.0	3.3	8.0	2.6	9.4	1.2	4.3	321
4	0.8	2.7	1.0	3.0	6.5	2.6	9.7	1.1	3.6	325
5	0.8	2.9	1.0	3.2	7.1	2.7	9.9	1.3	6.0	198
6	0.8	2.9	1.0	NC	5.9	2.6	9.7	1.3	3.7	327
Experimental results	0.8‡	2.8§	1.0‖	3.3‖¶	4.9	2.8	9.6‖	1.3‖	3.4	232

* Abbreviations used are TFE, 2,2,2-trifluoroethanol; PB, phenobarbital; TP450, total type P-450 cytochromes; XTP450, degraded or degradation of TP450; PB450, phenobarbital-induced form of cytochrome P-450; X450, degraded PB450; P448, cytochrome P-448; X448, degraded cytochrome P-448; and NC, not calculable.

† Reaction did not follow first-order kinetics. Where a numerical value is given in parentheses, the plot of $\ln(TFE_\infty - TFE_t)$ vs time was slightly curved and the value in parentheses represents an apparent k_{obs}, since the reaction was not strictly first order.

‡ From Ref. 10.

§ From Ref. 11.

‖ From Ref. 7.

¶ For XTP450.

Table 5. Compilation of rate constants utilized in the final runs of reaction schemes for the degradation by AIA of cytochrome P-450 in microsomes from phenobarbital-induced rats

Scheme	k_1	k_2	k_3	k_4	k_5	k_6	k_7	k_8	k_9
1	150	150	0.0	7.8	9	1.4			
1A	120	120	0.0	2	6	5.3			
2	100	100	0.0	70	50	50	1.5	63	
2A	100	100	0.0	8.4	50	50	2.0	72	
3	100	100	0.0	0.065	10	10	0.055		
4	100	100	0.0	10	10	0.1	10	10	0.053
5	100	100	0.0	10	10	0.14	10	10	0.06
6	100	100	0.0	0.06	0.009				

are not degraded by AIA or fluroxene and are not involved in the metabolism of fluroxene to 2,2,2-trifluoroethanol.

In conclusion, the reaction schemes for the degradation of cytochromes P-450 by AIA and fluroxene share several similarities. Suitable reaction schemes involve the production of reactive species from AIA and fluroxene by at least two forms of cytochrome P-450. The reactive species in question appear to degrade the same form of the enzyme (e.g. phenobarbital-induced cytochrome P-450, or 3-methylcholanthrene-induced cytochrome P-488) that produced the reactive species. It might be anticipated that the same enzyme molecule that activates fluroxene or AIA would subsequently be degraded by the activated metabolite that it produced, before that reactive species diffuses off the enzyme.

Reaction schemes involving the production of a reactive species by one form of cytochrome P-450 and the degradation of another form of cytochrome P-450 by that reactive species are not consistent with experimental data. It would appear, therefore, that the reactive metabolite mediating the degradation of cytochromes P-450 is a transient species. The observation that other vinyl ethers degrade the heme of cytochromes P-450, but that stable metabolites of fluroxene do not (see Results and Discussion), [20] is consistent with this conclusion.

Reaction schemes in which a bimolecular reaction of ferri- or ferrocytochrome P-450 (oxygenated or not) with AIA or fluroxene gives rise to the degradation of cytochromes P-450 do not fit the experimental data, inasmuch as it is not possible to characterize such reaction schemes with a K_M value.

On the basis of our investigations it was not possible to distinguish whether, for fluroxene, the same enzyme–substrate complex was involved in both the degradation of cytochromes P-450 and the production of 2,2,2-trifluoroethanol. It is possible that the enzyme–substrate complex giving rise to the degradation of cytochromes P-450 is identical to (scheme 3), in equilibrium with (scheme 4), or an entirely distinct complex from (scheme 5), that giving rise to the Type I difference spectrum for fluroxene and the production of 2,2,2-trifluoroethanol. Inasmuch as fluroxene does not produce a Type I difference spectrum of appreciable magnitude with cytochrome P-448 [10], and there is no spectral evidence for the binding of AIA to the substrate binding site of cytochromes P-450 with microsomes from

Table 6. Compilation of rate constants utilized in the final runs of reaction schemes for the degradation of cytochrome P-450 by fluroxene and for the production of 2,2,2-trifluoroethanol*

Scheme	Induction	k_1	k_2	k_3	k_4	k_5	k_6	k_7	k_8	k_9
1	PB	125	100	17	15	18	0.55			
1A	PB	125	100	16	1.7	5	5			
2	PB	125	100	17	80	50	40	5	85	
2A	PB	125	100	16	3	50	40	4	3	
3	PB	125	100	17	0.05	0.8	0.64	0.055		
4	PB	200	160	30	1	1	0.11	10	8	0.05
5	PB	200	160	37	10	8	0.08	10	8	0.045
6	PB	200	160	17	0.046	0.0015				
1	3-MC	50	40	14	13	17	2.5			
1A	3-MC	100	80	14	13	0.8	2.8			
2	3-MC	30	24	14	4.5	10	8	25	6.3	
2A	3-MC	40	32	14	0.41	10	8	10.5	1.4	
3	3-MC	40	32	14	0.025	0.04	0.032	0.1		
4	3-MC	40	32	15	0.1	10	3.5	0.045	0.036	0.1
5	3-MC	35	28	17	0.063	0.05	0.16	0.04	0.032	0.096
6	3-MC	30	24	14	0.02	0.0028				

* Abbreviations used are: PB, phenobarbital; and 3-MC, 3-methylcholanthrene.

differently pretreated rats [9], the latter possibility would appear to be favored for both fluroxene and AIA (see Results and Discussion).

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REFERENCES

1. F. De Matteis, *Biochem. J.* **124**, 767 (1971).
2. W. Levin, E. Sernatinger, M. Jacobson and R. Kuntzman, *Science* **176**, 1341 (1972).
3. W. Levin, M. Jacobson and R. Kuntzman, *Archs. Biochem. Biophys.* **148**, 262 (1972).
4. F. De Matteis, A. H. Gibbs and A. Unseld, *Biochem. J.* **168**, 417 (1977).
5. K. M. Ivanetich, J. A. Marsh, J. J. Bradshaw and L. S. Kaminsky, *Biochem. Pharmac.* **24**, 1933 (1975).
6. K. M. Ivanetich, J. J. Bradshaw, J. A. Marsh, G. G. Harrison and L. S. Kaminsky, *Biochem. Pharmac.* **25**, 773 (1976).
7. J. A. Marsh, J. J. Bradshaw, S. A. Lucas, L. S. Kaminsky and K. M. Ivanetich, *Biochem. Pharmac.* **26**, 1601 (1977).
8. P. R. Ortiz de Montellano, B. A. Mico and G. S. Yost, *Biochem. biophys. Res. Commun.* **83**, 132 (1978).
9. G. D. Sweeney and J. D. Rothwell, *Biochem. biophys. Res. Commun.* **55**, 798 (1973).
10. K. M. Ivanetich, J. J. Bradshaw, J. A. Marsh and L. S. Kaminsky, *Biochem. Pharmac.* **25**, 779 (1976).
11. J. J. Bradshaw, M. R. Ziman and K. M. Ivanetich, *Biochem. biophys. Res. Commun.* **85**, 859 (1978).
12. O. Tangen, J. Jonsson and S. Orrenius, *Analyt. Biochem.* **54**, 597 (1973).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. S. Chaykin, *Biochemistry Laboratory Techniques*, p. 20, John Wiley, New York (1966).
15. B. Stripp, N. Zampaglione, M. Hamrick and J. R. Gillette, *Molec. Pharmac.* **8**, 189 (1972).
16. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
17. H. Gion, N. Yoshimura, D. A. Holaday, V. Fiserova-Bergerova and R. E. Chase, *Anesthesiology* **40**, 553 (1974).
18. D. A. Blake, R. S. Rozman, H. F. Cascorbi and J. C. Krantz, Jr., *Biochem. Pharmac.* **16**, 1237 (1967).
19. H. F. Cascorbi and A. V. Singh-Amaranth, *Anesthesiology* **38**, 454 (1973).
20. K. M. Ivanetich, J. A. Marsh, J. J. Bradshaw and L. S. Kaminsky, in *Microsomes and Drug Oxidations* (Eds. V. Ullrich, I. Roots, A. Hildebrandt, R. W. Estabrook and A. H. Conney), p. 76, Pergamon Press, Oxford (1977).
21. W. Levin and R. Kuntzman, *J. biol. Chem.* **244**, 3671 (1969).
22. A. F. Welton and S. D. Aust, *Biochem. biophys. Res. Commun.* **56**, 898 (1974).
23. M. D. Burke and R. T. Mayer, *Drug Metab. Dispos.* **2**, 583 (1974).
24. A. Y. H. Lu, W. Levin, D. Ryan, S. B. West, P. Thomas, J. Kawalek, R. Kuntzman and A. H. Conney, in *Anticonvulsant Drugs and Enzyme Induction* (Eds. A. Richens and P. F. Woodford), p.169, Associated Scientific Publishers, Amsterdam, NY (1976).
25. R. A. Prough, V. W. Patrizi and R. W. Estabrook, *Cancer Res.* **36**, 4439 (1976).
26. D. Ryan, A. Y. H. Lu, J. Kawalek, S. B. West and W. Levin, *Biochem. biophys. Res. Commun.* **64**, 1134 (1975).
27. A. F. Welton, F. O. O'Neal, L. C. Chaney and S. D. Aust, *J. biol. Chem.* **250**, 5631 (1975).