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ETHOXY-, PENTOXY- AND BENZYLOXYPHENOXAZONES AND HOMOLOGUES: A SERIES OF SUBSTRATES TO DISTINGUISH BETWEEN DIFFERENT INDUCED CYTOCHROMES P-450

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Abstract—The individual members of a homologous series of phenoxazone ethers related to ethoxyresorufin were *O*-dealkylated, and the parent compound phenoxazone was ring-hydroxylated, each at different rates with hepatic microsomes of untreated rats. A structure–activity relationship (SAR) was plotted, relating the rate of *O*-dealkylation to the length and type of the ether side-chain. Phenobarbitone (PB), 3-methylcholanthrene (MC), Aroclor 1254 (ARO), isosafrole (ISO) and SKF-525A each induced preferentially the *O*-dealkylation of different members of the homologous series, resulting in the appearance of 5 different SAR plots, which characterized and differentiated between the 5 different inducers. β-Napthoflavone (BNF) had a similar effect to MC, whereas pregnenolone 16α-carbonitrile treatment caused no large change in the metabolism of any of the substrates tested. For characterizing the effects of the different inducers it was largely sufficient to compare the *O*-dealkylations of just 4 of the ethers: methoxy-, ethoxy-, pentoxy- and benzyloxyphenoxazone. Very high degrees of induction were seen. MC and ARO each induced preferentially the *O*-dealkylation of ethoxyphenoxazone (51- and 61-fold respectively). PB and SKF-525A each induced preferentially the *O*-dealkylation of pentoxyphenoxazone (283- and 324-fold respectively). ISO induced preferentially the *O*-dealkylation of benzyloxyphenoxazone (43-fold). For any particular induced type of microsomes the substrate with the fastest metabolism was not necessarily the substrate whose metabolism was induced the most, so that in order to characterize each of the 5 different inducers (PB, MC/BNF, ARO, ISO, SKF) it was necessary to compare both the degrees of induction and the specific activities of the reactions. Experiments with purified cyt. P-450 isozymes showed that ethoxyphenoxazone and pentoxyphenoxazone were highly selective substrates for the major isozymes induced by MC and PB respectively, whilst benzyloxyphenoxazone was a good substrate for both isozymes. Experiments using the organic inhibitors metyrapone and α-naphthoflavone and inhibitory antibodies against individual cyt. P-450 isozymes indicated that similar substrate selectivities occurred with the monooxygenase system in the microsomal membrane. It is suggested that the use of some or all of these homologous phenoxazone ethers will provide both a simple routine test for the characterization of several types of inducing agents and a powerful tool for investigating the biochemical basis for cyt. P-450 isozyme substrate selectivity.

Inducers of the Phase 1 (cyt. P-450-mediated) reactions of drug metabolism have long been categorized into different types [1]. One of the chief differentiating criteria is that each type of inducer affects a different spectrum of drug-metabolizing enzyme reactions. The primary explanation for this is that each type of inducer increases the synthesis of a characteristic range of cyt. P-450 isozymes, with each isozyme showing a distinctive, but not necessarily unique, substrate and reaction specificity [2]. The accurate identification and quantitation of induced cyt. P-450 isozymes is important for both the study of the induction process and the categorization of new inducers. Despite the advent of immunological and recombinant-DNA methods for identifying individual induced isozymes of cyt. P-450, identification and quantitation by measuring the metabolism of isozyme-selective substrates probably remains for most laboratories at the moment a more practicable approach. A total reliance on immunological and recombinant-DNA methods is, moreover, of questionable validity in view of increasing evidence that microsomes contain variable proportions of enzymically inactive yet antigenic cyt. P-450 apoproteins [3, 4]. Unfortunately, many of the "model" substrates widely used for measuring cyt. P-450 induction do not appear to differentiate adequately between individual induced isozymes, nor between induced and constitutive isozymes [3, 5]. For example, aminopyrine N-demethylation and aldrin epoxidation are reactions that are generally considered to be selective for PB-induced cyt.

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P-450, yet their microsomal activities are induced only 3- to 6-fold by PB,* despite a 40- to 60-fold increase in the amount(s) of the PB-induced isozyme(s) as measured immunochemically [3, 6, 7]. One very probable explanation is that these reactions are catalysed extensively by constitutive isozymes as well as by PB-induced isozymes [3]. There are, however, a small number of cyt. P-450 reactions, whose microsomal activities can not only be induced as extensively as immunochemically-measured cyt. P-450, but also display a high degree of inducer selectivity (i.e. the reactions are greatly induced by some inducers but not by others). Ethoxyphenoxazone (ethoxyresorufin) *O*-de-ethylation is an example of such a reaction: in the hepatic microsomes of rats and mice its specific activity is induced 50- to 70-fold by MC, whilst several other classes of inducer, e.g. PB and PCN, induce it less than 3-fold [8, 9]. This reaction has, accordingly, been shown to be highly selective for the major MC- or BNF-induced isozyme of rat liver cyt. P-450 [2, 5, 10], the amount of which, as determined immunochemically in rat liver microsomes, is similarly induced 40- to 50-fold by BNF [2, 3, 7]. In mice the pentyl homologue of ethoxyresorufin, pentoxyphenoxazone, appears also to be highly selective, but in this case for the PB-induced isozymes(s) of cyt. P-450 [9]. We report here a number of homologues of ethoxyphenoxazone, whose *O*-dealkylations display high degrees of induction, selectivity for certain purified cyt. P-450 isozymes, and the apparent ability to distinguish between the effects of a number of different types of inducers.

MATERIALS AND METHODS

Animals and pretreatments

Male 250–300 g Sprague–Dawley rats, bred at the University of Aberdeen, were housed on Sorbitex mineral bedding (J. Strachan, Perth) and fed Oxoid Breeding Diet and water *ad lib.* Drug treatments were as follows (all treatments being ceased 24 hr before death, except for MC, which was given 72 hr before the rats were killed): PB 0.1% in the drinking water for 6 days; MC (1% in olive oil) 80 mg/kg i.p. once; BNF (1% in olive oil) 80 mg/kg i.p. daily for 3 days; ARO (20% in olive oil) 250 mg/kg i.p. on day 1 then 25 mg/kg i.p. daily for 5 more days; ISO (10% in olive oil) 200 mg/kg i.p. daily for 3 days; SKF-525A (2.5% in 0.9% NaCl) 50 mg/kg i.p. daily for 3 days; PCN (5% in 5% Tween 80) 50 mg/kg p.o. daily for 4 days. Control treatments with appropriate drug vehicles were also carried out.

Chemicals

Sources of chemicals were as follows: PB, BDH Ltd. (Poole, U.K.); MC, Fluka AG (CH-9470 Buchs, Switzerland); ANF, BNF and metyrapone, Aldrich Chemical Co. (Gillingham, U.K.); NADPH, EDTA and HEPES, Sigma Chemical Co. (Poole, U.K.); ARO and ISO, ICI plc. (Alderley Park, U.K.); SKF was a generous gift from Smith Kline & French (Welwyn Garden City, U.K.); PCN was kindly provided by Dr M. J. Tredger, Kings College Hospital, London. Phenoxazone and its *n*-alkyl and aryl ethers were synthesized as described elsewhere [9]. Resorufin (hydroxyphenoxazone) and the methyl-, ethyl-, pentyl- and benzyl- ethers of phenoxazone can also be obtained from Molecular Probes Inc. (24750 Lawrence Road, Junction City, Oregon 97448, U.S.A.).

Biological preparations

(a) *Liver microsomes.* These were prepared by differential centrifugation involving KCl-washing as described elsewhere [11].

(b) *Purified cytochromes P-450 and NADPH-cyt. P-450 reductase.* The major PB-induced isozyme ($P\text{-}450_{PB\text{-}B2}$) and the major BNF-induced isozyme ($P\text{-}450_{BNF\text{-}B2}$) of cyt. P-450 were purified from livers of PB- and BNF-treated rats respectively, whilst NADPH-cyt. P-450 reductase was purified from livers of PB-treated rats, as described elsewhere [12]. The cyt. P-450 fractions each gave a single band on SDS–polyacrylamide gel electrophoresis and their specific contents were each 15 nmoles per mg protein. The specific activity of the NADPH-cyt. P-450 reductase was 44 μmoles cyt. *c* reduced per min per mg protein as assayed in 300 mM K-phosphate buffer, pH 7.7 at 25°. Purified enzymes were stored at −80°.

(c) *Antibodies.* Antibodies were raised against the purified cyt. P-450 isozymes in adult male New Zealand rabbits. Rabbits were bled prior to immunization to obtain pre-immune sera. The methods for raising the antibodies and for isolating IgG fractions by affinity chromatography on Protein A-Sepharose CL-4B columns are described elsewhere [12]. The IgG fractions were stored at −80°.

O-*Dealkylation reactions*

(a) *Using microsomes.* Either 20 μg or 200 μg of microsomal protein were used in the reactions, being varied according to the substrate and the type of induction in order to ensure linear reaction rates for at least 5 min. The most appropriate amounts of microsomal protein could only be determined by trial and error, but in general 20 μg protein was suitable for all but the slowest reactions. Reactions were carried out in fluorimeter cuvettes at 37° using a Perkin-Elmer Model 3000 spectrofluorimeter. The reaction mixture, containing microsomal protein (see above), 5 μM substrate (10 μl of a 1 mM solution in DMSO) and 0.1 M phosphate buffer (Na–K salts, pH 7.6), was equilibrated for 1 min at 37°. The reaction was then started by the addition of 250 μM NADPH (10 μl of a 50 mM solution). The final reaction volume was 2 ml. The reaction rate was measured directly by the increasing fluorescence of the reaction mixture as displayed on a chart recorder. Evidence is presented elsewhere [8, 9] that the increase in reaction fluorescence is entirely due to the cyt. P-450-dependent formation of a single

* Abbreviations used: Ab, antibody; ANF, α-naphthoflavone; ARO, Aroclor 1254; BNF, β-naphthoflavone; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; ISO, isosafrole; MC, 3-methylcholanthrene; PB, phenobarbitone; PCN, pregnenolone 16α-carbonitrile; SAR, structure–activity relationship; SKF-525A, β-diethylaminoethyl diphenyl valerate.

metabolite, resorufin, from each of the substrates used. The fluorimeter settings were: excitation and emission slits, 5 nm; excitation and emission wavelengths, 530 and 585 nm respectively. After a suitable period of reaction the extent of fluorescence increase was calibrated by the addition to the cuvette of a 10 μl aliquot of authentic resorufin (10 or 25 μM in DMSO, the concentration chosen being dependent on the sensitivity setting of the fluorimeter). *O*-Dealkylation reactions and cyt. P-450 measurements with microsomes from SKF-525A-induced rats were measured both before and after dissociation of the SKF-525A-cyt. P-450 complex, achieved by preincubation of the microsomes with 50 μM ferricyanide at 37° for 3 min [13]. Cyt. P-450 measurements with microsomes from ISO-induced rats were measured both before and after dissociation of the ISO-cyt. P-450 complex, achieved by preincubation of the microsomes with 250 μM 2-*n*-heptyl benzimidazole at 37° for 20 min [14]. However, neither prior nor concomitant dissociation of the ISO-cyt. P-450 complex by either heptyl benzimidazole or any other extra agent was carried out in the case of the measurements of *O*-dealkylation reactions with ISO-induced microsomes.

(b) *Using purified cyt. P-450.* Reactions were carried out at 37° as for microsomes, with the following changes. The total reaction volume was 1 ml. A 20 pmole quantity (10 μl) of purified cyt. P-450 was mixed with 0.03 units (10 μl) of NADPH-cyt. P-450 reductase (1 unit reduced 1 μmol cyt. *c* per min), 25 μg dilauroylphosphatidylcholine (10 μl of freshly sonicated solution in HEPES buffer), 100 μg sodium deoxycholate (10 μl of solution in HEPES buffer) and 60 μl HEPES buffer (0.05 M, pH 7.6, containing 0.1 mM EDTA and 15 mM $MgCl_2$). This mixture was equilibrated at 37° for 2 min, then substrate (10 μl of a 1 mM solution in DMSO; final substrate concentration 5 μM) and 0.88 ml HEPES–EDTA–$MgCl_2$ buffer were added. The reaction was started with NADPH (10 μl of a 50 mM solution; final concentration 250 μM) and calibrated with authentic resorufin as above. HEPES–EDTA–$MgCl_2$ buffer was used for these reactions because it had been used in all previous studies with the purified cyt. P-450 preparations used here [12].

Inhibition studies

These were carried out using only ARO-induced rat liver microsomes and only ethoxy-, pentoxy- and benzyloxyphenoxazone as substrates.

(a) *Organic inhibitors.* Standard microsomal reactions were run with the addition of either metyrapone or ANF. Final concentrations of these inhibitors were either 10^{-6} M or 10^{-5} M, obtained by adding 10 μl of either 0.2 mM or 2 mM solutions in DMSO per 2 ml reaction. The effects of 10 μl DMSO were also measured.

(b) *Antibodies.* IgG fractions of antibodies against individual cyt. P-450 isozymes were incubated with 20 μg of ARO-induced microsomes (containing 2.65 nmol total cyt. P-450 per mg protein) in a total volume of 0.1 ml Na–K phosphate buffer (0.1 M, pH 7.6) at room temperature for 20 min. Antibodies were mixed with microsomes in the ratios of either 10:1, 25:1 or 53:1 mg Ab per mg microsomal protein, equivalent to ratios of 3.8:1, 9.4:1 and 20:1 mg Ab per nmol microsomal total cyt. P-450. The mixture was then placed on ice for up to 1 hr before use in a reaction. It was checked that reaction rates were not altered by this period on ice. After preincubation the antibody–microsome mixture was transferred to a cuvette and a standard microsomal *O*-dealkylation reaction carried out as described above. Control reactions, either with the addition of IgG fraction from pre-immune serum or without any additions, were also carried out.

Other assays

Total microsomal concentrations of cyt. P-450 were measured by the ferrous carbonyl difference spectroscopic method of Omura and Sato [15]. Protein was measured by the Lowry assay [16], using bovine serum albumin as the standard.

RESULTS

Effects of inducers on microsomal O*-dealkylation of alkoxyphenoxazones*

The different members of a homologous series of phenoxazone ethers were not all metabolized at the same rate by rat liver microsomes. A structure–activity relationship was constructed, relating the rate of metabolism to the length and the type of the ether side-chain. Induction of cyt. P-450 caused a change in the SAR. Figure 1 is a collection of SAR plots in histogram form, showing the effects of the inducers PB, MC, BNF, ARO, SKF-525A, ISO and PCN respectively, on the rat hepatic microsomal *O*-dealkylation of a homologous series of *n*-alkyl ethers of phenoxazone, with side-chain lengths from 1C (methoxyphenoxazone) to 8C (octoxyphenoxazone). Also shown are the effects of the inducers on the aryl hydroxylation of the parent compound, phenoxazone (side-chain length = 0), and on the *O*-debenzylation of its benzyl ether (benzyloxyphenoxazone; side-chain length designated by the letter B). For reasons explained below, attention has been focused on ethoxy-, pentoxy- and benzyloxyphenoxazone by highlighting their bars with extra-thick lines. The corresponding results for the appropriate vehicle-treated control rats are overlaid on each histogram as solid bars. The SAR plots for the various vehicle treatments were not all identical. The effects of each inducer can most easily be seen by comparing the induced SAR plot with the vehicle-control SAR plot. This comparison shows (a) which substrates were metabolized the fastest and (b) for which substrates the metabolism was induced the most. It is important to realize that in the microsomes from a rat treated with a particular inducer, the substrate showing the highest rate of metabolism was not necessarily the substrate showing the greatest degree of induction over controls. For example, in PB-induced microsomes methoxy-, ethoxy-, butoxy- and pentoxyphenoxazone were all metabolized at similar rates and were, after benzyloxyphenoxazone, the fastest substrates, but the induction of the metabolism of pentoxyphenoxazone (283-fold) was an order of magnitude greater than the inductions of the 1C–4C ethers (6.5- to 36-fold). A comparison of

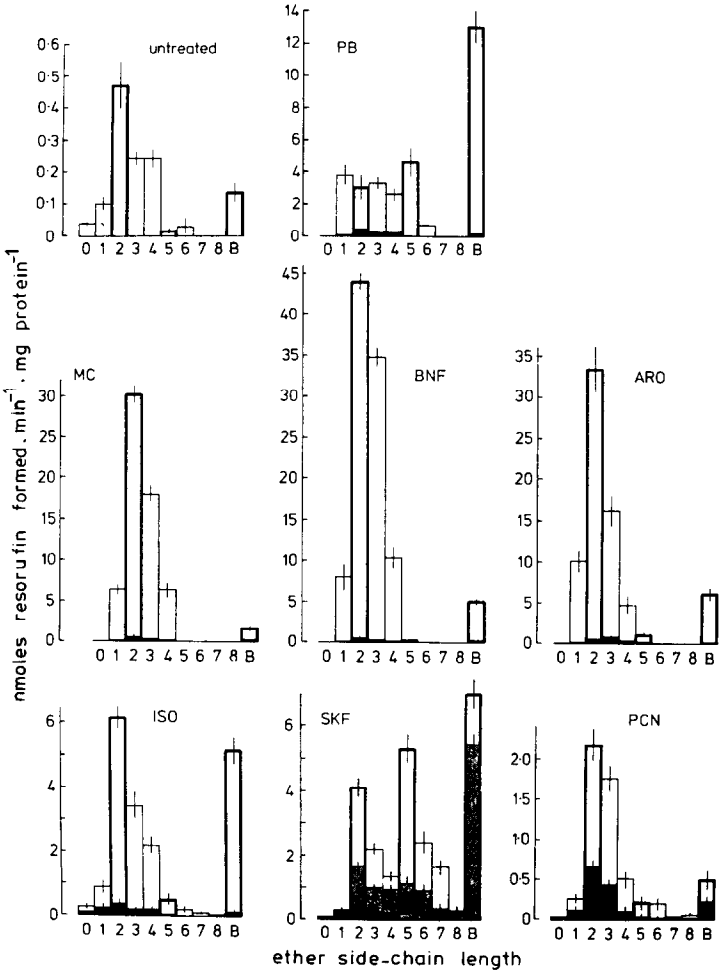

Fig. 1. Effects of induction on the microsomal *O*-dealkylation of phenoxazone and a homologous series of its ethers. The histograms illustrate structure–activity relationships relating the rate of *O*-dealkylation to the length and type of the ether side-chain. The numbers beneath the histogram bars indicate the number of carbon atoms in the *n*-alkyl ether side-chain. Number 0 represents the parent compound, phenoxazone, which did not have a side-chain and which underwent ring-hydroxylation at the same position as the *O*-dealkylation undergone by the ethers. The letter B represents the benzyl ether. The titles above each histogram indicate the inducer given to the rats: untreated; PB, phenobarbitone; MC, 3-methylcholanthrene; BNF, β-naphthoflavone; ARO, Aroclor 1254; ISO, isosafrole; SKF, SKF-525A; PCN, pregnenolone 16α-carbonitrile. For all but the untreated and SKF-525A-treated rats there are two histograms overlaid: the solid bars are for rats treated with the appropriate drug vehicle and the open bars are for the rats treated with the inducer drug. For the SKF-525A-treated rats there are three histograms overlaid: the solid bars are vehicle controls, whilst the cross-hatched and open bars are for SKF-treated rats before and after ferricyanide-displacement of the microsomal SKF-525A-cyt. P-450 complex respectively. All bars start from an ordinate value of zero. Note that a number of different ordinate scales have been used. Results are means ± S.D. for three individual rats. Treatment schedules are detailed in the Methods.

the different shapes of the SAR plots for induced microsomes affords a clear picture of the differences between the effects of the inducers.

In control microsomes the ethyl ether was the best substrate (i.e. it gave the fastest reaction), followed by the other short-chain ethers up to butyl, and the benzyl ether. In PB-induced microsomes the benzyl ether was the best substrate, followed by the short-chain ethers (methyl to pentyl, which were all metabolized at approximately equal rates). In either MC-, BNF- or ARO-induced microsomes the ethyl and propyl ethers were the best substrates, followed by the other short-chain ethers (methyl and butyl) and the benzyl ether. There was a much greater difference in the MC- and BNF-induced microsomes than in any of the other induced types of microsomes between the reaction rates of the best substrates and the rates of the next-best substrates. In ISO-induced microsomes the ethyl and benzyl ethers were equally the best substrates, followed by the propyl and butyl ethers. In SKF-525A-induced microsomes that had not had the SKF-525A-cyt. P-450 complex dissociated the benzyl ether was clearly the best substrate. However, the benzyl, pentyl and ethyl

ethers were all much better substrates than the other homologues in SKF-525A-induced microsomes that had had the complex dissociated. Complexation of cyt. P-450 by SKF-525A appeared to inhibit preferentially the metabolism of the ethyl and pentyl ethers, with much less effect on the benzyl or other ethers. In PCN-induced microsomes the SAR plot was similar to that for control microsomes, albeit with evidence of a small induction of all the reactions without preference. The pentyl ether was a good substrate only with PB- and SKF-525A-induced microsomes, whereas with control and all other induced microsomes it was among the poorest substrates. The parent compound of the series, phenoxazone, which underwent 7-hydroxylation, was a poor substrate for all the different types of microsomes tested.

The effects of the inducers can be clearly differentiated by a comparison of just three of the substrates: ethoxy-, pentoxy- and benzyloxyphenoxazone. Figure 2 portrays the two important aspects of this comparison. First, it shows sets of 3-bar histograms (called EPB-profiles) for the differently induced microsomes, where the bar heights indicate the relative rates of metabolism of the three substrates, calculated in each case as percentages of the rate of whichever was the fastest of the three reactions in that particular induced type of microsomes. Secondly, it lists the fold-inductions of the dealkylations of the three substrates by each inducer. Based on Fig. 2, the differences between the effects of the inducers can be summarized as follows:

PB-induction was characterized by a high relative reaction rate with only benzyloxyphenoxazone and by large inductions of both the dependentylation and the debenzylation reactions, with a preferential induction of dependentylation (283-fold) compared to debenzylation (95-fold).

MC-induction was characterized by a high relative reaction rate with only ethoxyphenoxazone and a large induction of only the de-ethylation reaction (51-fold).

The characteristics of BNF-induction were virtually the same as for MC-induction.

Aroclor 1254 reportedly combines many of the inducing characteristics of both PB and MC [17, 18]. This was in part borne out in this study, but the MC-type inducing effect of ARO (induction of ethoxyphenoxazone de-ethylation) considerably exceeded its PB-type effect (induction of pentoxy- and benzyloxyphenoxazone metabolism). Thus, whilst expectations that the highest relative reaction rate would be with ethoxyphenoxazone were borne out, and while the actual rate of the de-ethylation reaction was virtually the same after ARO-treatment as after induction by MC, the relative and actual rates of metabolism of pentoxy- and benzyloxyphenoxazone in ARO-induced microsomes were only 12–50% of those expected from the results of induction by PB. Whereas ARO was slightly more effective at inducing de-ethylation (61-fold) than was MC (51-fold), it was much less effective than PB at inducing either dependentylation (22-fold compared to 283-fold by PB) or debenzylation (30-fold compared to 95-fold by PB). It was noticeable that the difference between the inducing effects of ARO and PB was much greater with respect to dependentylation than with respect to debenzylation.

A common feature of PB-, MC-, BNF- and ARO-induced microsomes was that methoxyphenoxazone

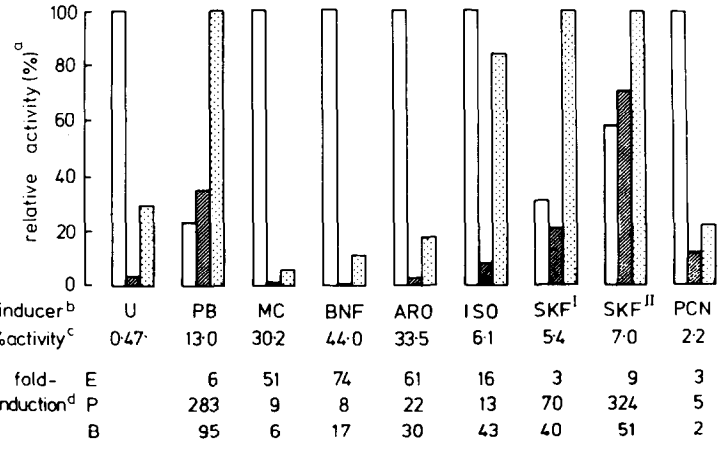

Fig. 2. EPB-profiles illustrating the different effects of inducers on the rates of microsomal *O*-dealkylation of ethoxy-, pentoxy- and benzyloxyphenoxazone. (a) Relative activity is the rate of *O*-dealkylation of each ether expressed as a percentage of the rate for whichever was the fastest of the three ethers with that particular induced type of microsomes (the rate of the fastest = 100%). (b) The letters under the groups of bars indicate the inducer given to the rats. U = untreated and the other letters are as for Fig. 1. SKF[I] and SKF[II] illustrate respectively reactions carried out with and without prior ferricyanide-dissociation of the SKF 525A–cyt. P-450 complex, as described in the Methods. (c) These values are the specific activities of the fastest of the three reactions for each type of microsomes (nmol resorufin produced · min^{-1} · mg $protein^{-1}$). (d) These values represent the extent of the induction of the metabolism of either ethoxy- (E), pentoxy- (P) or benzyloxy- (B) phenoxazone by the inducer indicated in line (b). The fold-induction is the ratio for the specific activity of the microsomal reaction between induced and untreated rats.

was metabolized at appreciably higher-than-control rates (Fig. 1). In contrast, with either PCN-, ISO- or SKF-525A induced microsomes (the latter either with or without ferricyanide dissociation—see below), methoxyphenoxazone was metabolized at virtually control rates. Consequently, it would probably be worthwhile including methoxyphenoxazone along with the ethoxy, pentoxy and benzyloxy homologues in an activity screen used to characterize an inducing agent.

The effects of ISO-induction were different from either PB- or MC-induction (Fig. 2). It should be noted that ISO forms a stable complex with the induced cyt. P-450 [14] and that, whereas this complex was dissociated with 2-*n*-heptylimidazole prior to the spectroscopic measurement of cyt. P-450, phenoxazone ether metabolism was measured in the absence of 2-*n*-heptylimidazole and without prior dissociation of the complex. ISO was characterized by high relative reaction rates with both ethoxy- and benzyloxyphenoxazone and by a preferential induction of the debenzylation reaction (43-fold compared with 16- and 13-fold for de-ethylation and depentylation respectively). Other differences between ISO-induced and either MC- or PB-induced microsomes were as follows: in ISO-induced microsomes, (a) the rate of debenzylation was three times that in MC-induced microsomes but only 39% of that in PB-induced microsomes, (b) the rate of de-ethylation was only 20% of that in MC-induced microsomes, and (c) the rate of depentylation was only 10% of that in PB-induced microsomes. Since the presumed ISO-cyt. P-450 complex was not dissociated with 2-*n*-heptylimidazole, it may seem surprising that ISO-induced *O*-dealkylation reactions occurred. However, the results of Fisher *et al.* [19] suggest that the alkylimidazole-dissociable ISO-complex may not inhibit reactions of ISO-induced microsomes other than the metabolism of ISO itself. Alternatively, those phenoxazone ethers that were the best substrates for ISO-induced microsomes may themselves have dissociated the ISO-complex; although we have not tested this, Dickins *et al.* [14] showed that a number of cyt. P-450 substrates are able to do this.

SKF-525A induces cyt. P-450 but also gives rise to a stable inhibitory complex with the induced cyt. P-450 [13], which it is often necessary to dissociate in order to see the effects of induction. The metabolism of the phenoxazone ethers by SKF-525A-induced microsomes was measured both with and without prior dissociation of the complex with ferricyanide. Although, as for the other inducers, a comparison of ethoxy-, pentoxy- and benzyloxyphenoxazone is the best means of characterizing the effects of SKF-525A, in the case of SKF-525A it is instructive to also look at the complete SAR plot (Fig. 1) in addition to the EPB-profile (Fig. 2). The complete SAR plot for ferricyanide-dissociated, SKF-525A-induced microsomes (Fig. 1) was distinctive in showing three clear peaks, at (in descending order of reaction rates) benzyloxy-, pentoxy- and ethoxyphenoxazone. These reactions were, however, each slower than when induced by whichever was the most effective of PB, MC, BNF or ARO. As depicted in Fig. 2, SKF-525A (after ferricyanide-dissociation) could be described as being similar to PB, but with the rates of de-ethylation and depentylation being considerably faster, relative to the rate of debenzylation, with SKF-525A than with PB. SKF-525A (after ferricyanide-dissociation) was further similar to PB in that the greatest induction occurred for the depentylation reaction (324-fold) and that debenzylation was also considerably induced (51-fold). A characteristic feature of SKF-525A-induction was that there was extensive induction of the dealkylation of hexoxy- and heptoxyphenoxazone, resulting in these reactions being much faster in ferricyanide-dissociated SKF-525A-induced microsomes than in the other induced types of microsomes (Fig. 1). The effects described above are all for ferricyanide-dissociated microsomes. In the absence of ferricyanide-dissociation the rates of metabolism of ethoxy- and pentoxyphenoxazone were much slower than after dissociation, but, interestingly, benzyloxyphenoxazone was metabolized almost as fast without as with prior ferricyanide-dissociation.

PCN did not cause very much induction of the metabolism of any of the substrates tried and the shapes of the SAR plot and EPB-profile for PCN-induced microsomes were virtually the same as for microsomes of untreated rats. It was as if PCN had caused a small and virtually non-selective induction of the metabolism of the whole series of phenoxazone ethers.

Alkoxyphenoxazone dealkylation by purified cytochrome P-450

Figure 3 shows the alkoxyphenoxazone specificities of purified cyt. P-$450_{PB\text{-}B2}$ (the major PB-induced isozyme) and purified cyt. P-$450_{BNF\text{-}B2}$ (the major BNF-induced isozyme) in reconstituted monooxygenase systems. The two isozymes showed clearly differing specificities, with the principle differences involving ethoxy-, propoxy-, pentoxy- and benzyloxyphenoxazone. Ethoxy- and propoxyphenoxazone were highly selective substrates for cyt. P-$450_{BNF\text{-}B2}$, pentoxyphenoxazone was highly selective for cyt. P-$450_{PB\text{-}B2}$, and benzyloxyphenoxazone was a good substrate for both isozymes, but with a clear selectivity for cyt. P-$450_{PB\text{-}B2}$. These results for purified cyt. P-450 correspond very well with the results for the effects of PB- and BNF- (or MC-) induction on alkoxyphenoxazone dealkylation by microsomes, and confirm that induced changes in the alkoxyphenoxazone specificities of microsomes are probably due to changes in the population of cyt. P-450 isozymes that is present.

Effects of inhibitors on alkoxyphenoxazone dealkylation by Aroclor 1254-induced microsomes

Because there will always be doubt as to how accurately the specificities of individual purified cyt. P-450 isozymes reflect the specificities of the native membrane-bound isozymes present in a mixed population [20], we further investigated the alkoxyphenoxazone specificities of cyt. P-450 in microsomes by using supposedly isozyme-selective organic inhibitors and inhibitory antibodies. We used metyrapone and ANF as selective inhibitors of PB-induced and MC- (or BNF-) induced cyt. P-450 respectively [21], and we also used inhibitory antibodies against cyt. P-

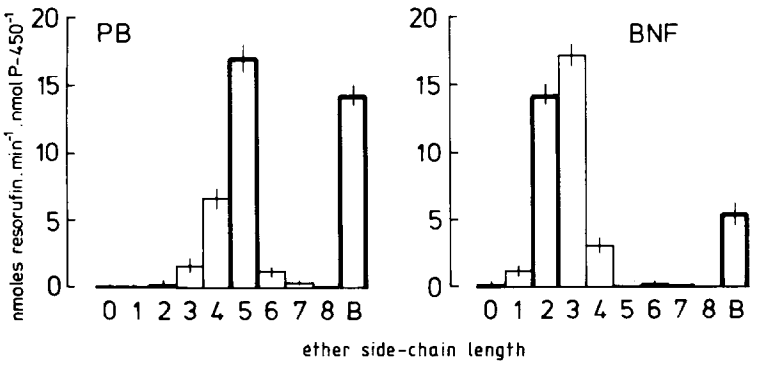

Fig. 3. Different substrate selectivities of purified phenobarbitone-induced and β-naphthoflavone-induced cytochrome P-450 isozymes for a homologous series of phenoxazone ethers. The numbers beneath the bars indicate the number of carbon atoms in the *n*-alkyl ether side-chain. Number 0 represents the parent compound, phenoxazone. The letter B represents the benzyl ether. Results are means ± S.D. for three determinations with each isozyme: PB = the major phenobarbitone-induced isozyme (P-450$_{PB-B2}$); BNF = the major β-naphthoflavone-induced isozyme (P-450$_{BNF-B2}$). The bars for the ethyl- (2), pentyl- (5) and benzyl- (B) ethers have been highlighted by extra-thick lines because these are the ethers that most clearly differentiate between the microsomal effects of different inducers.

450$_{PB-B2}$ (the major PB-induced isozyme) and against cyt. P-450$_{BNF-B2}$ (the major BNF-induced isozyme). These organic and immunological inhibitors were applied to the dealkylation of ethoxy-, pentoxy- and benzyloxyphenoxazone by ARO-induced microsomes. We used ARO-induced microsomes because they contain both PB-induced and MC- (or BNF-) induced isozymes of cyt. P-450 and showed evidence of both PB-type and MC- (or BNF-)type induction of alkoxyphenoxazone dealkylation (see above). In this part of the study we confined ourselves to ethoxy-, pentoxy- and benzyloxyphenoxazone because, as described above, these are the substrates that most clearly differentiate between PB-induced and MC- (or BNF-) induced microsomes. The results are shown in Table 1. DMSO, the solvent for both metyrapone and ANF, had little effect on the reactions at the concentration used (10 μl DMSO per 2 ml). Metyrapone (1 μM) selectively inhibited the dealkylation of pentoxy- and benzyloxyphenoxazone and had no effect on the dealkylation of ethoxyphenoxazone. Conversely, ANF (1 μM) selectively inhibited the dealkylation of ethoxyphenoxazone and had a much lesser inhibitory effect on the dealkylation of pentoxy- and benzyloxyphenoxazone. The IgG fraction of preimmune serum had

Table 1. Effects of organic inhibitors and of antibodies against cyt. P-450 on alkoxyphenoxazone *O*-dealkylation by hepatic microsomes from Aroclor 1254-induced rats*

Addition to reaction	Activity (% of control)†		
	Ethoxy-	Pentoxy-	Benzyloxy-
DMSO‡	100 ± 9	80 ± 5	84 ± 3
Metyrapone§	100 ± 7	46 ± 3	54 ± 4
ANF§	2 ± 0.5	52 ± 4	41 ± 3
Preimmune IgG‖	102 ± 8	86 ± 5	99 ± 7
Anti-P-450$_{PB-B2}$‖	72 ± 6	21 ± 3	18 ± 3
Anti-P-450$_{BNF-B2}$‖	7 ± 2	70 ± 5	43 ± 4

* Aroclor 1254 treatment was as in Materials and Methods.

† 100% control activities (standard reactions with no additions) were (nmol resorufin produced · min^{-1} · mg protein^{-1}): ethoxyphenoxazone *O*-de-ethylation, 33.5; pentoxyphenoxazone *O*-depentylation, 4.8; benzyloxyphenoxazone *O*-debenzylation, 6.1. Reactions were measured as in Methods. Results are means ± S.D. for 3 individual rats.

‡ 10 μl DMSO per 2 ml reaction.

§ Either metyrapone or ANF were added (in 10 μl DMSO per 2 ml reaction) to a final concentration of 10^{-6} M.

‖ Either preimmune IgG, or the IgG fraction of antibodies against either cyt. P-450$_{PB-B2}$ or cyt. P-450$_{BNF-B2}$, was added to reactions at a ratio of 20 mg IgG per nmol total microsomal ferrous carbonyl cyt. P-450 (=53 mg IgG per mg microsomal protein).

little effect on the reactions. At a 20:1 ratio (mg antibody per nmol total microsomal cyt. P-450) the antibody against cyt. P-450$_{PB-B2}$ preferentially inhibited the dealkylation of pentoxy- and benzyloxyphenoxazone. At the same ratio the antibody against cyt. P-450$_{BNF-B2}$ preferentially inhibited the dealkylation of ethoxyphenoxazone and was much more inhibitory toward the dealkylation of benzyloxyphenoxazone than toward the dealkylation of pentoxyphenoxazone.

These results with metyrapone, ANF and the antibodies are in accordance with an interpretation that ethoxyphenoxazone is highly selective for (certain) MC- (or BNF-)induced isozyme(s) of cyt. P-450, that pentoxyphenoxazone is highly selective for (certain) PB-induced isozyme(s) of cyt. P-450, that benzyloxyphenoxazone is metabolized by both PB- and MC- (or BNF-)induced isozymes, but preferentially by PB-induced isozyme(s), and that ARO induces (certain) MC- (or BNF-)inducible isozyme(s) to a greater extent than it induces (certain) PB-inducible isozyme(s).

DISCUSSION

We have aimed to show here that a homologous series of phenoxazone ethers related to ethoxyresorufin, and in particular the methyl, ethyl, pentyl and benzyl ethers, can be used as substrate probes to characterize and differentiate between a variety of inducers of rat liver cyt. P-450. The key property that distinguishes these substrates from the majority of other substrates used to measure induction of cyt. P-450 is the very high degrees of induction of the microsomal metabolism of certain phenoxazone ethers: up to 320-fold as compared to only about 3- to 5-fold for most other commonly used "model" substrates, e.g. aminopyrine, benzphetamine, aldrin, biphenyl and ethoxycoumarin. The relatively poor extent of induction as measured using these latter substrates at the microsomal level contrasts with the fact that they show very large differences in activity between different purified induced isozymes of cyt. P-450 [2]. The probable explanation is that ethoxy-, pentoxy- and benzyloxy-phenoxazone show high substrate selectivity for certain induced isozymes of cyt. P-450 and are probably very poor substrates for the basal or constitutive isozymes that make up the bulk of the cyt. P-450 present in the liver microsomes of control rats, whereas the other substrates listed above probably react almost as well with constitutive isozymes as they do with the induced isozymes for which they are supposedly selective. It is only during the past few years, in fact, that it has become realized, as a result of the use of immunoquantitation techniques, that the levels of individual cyt. P-450 isozymes can increase in the liver microsomes by as much as 50-fold or more in response to inducers [3, 22]. With ethoxy-, pentoxy- and benzyloxyphenoxazone, plus a small number of other substrates, for example phenprocoumon [23] and dichloro-*p*-nitroanisole [24], one is at last seeing induced increases in microsomal cyt. P-450 isozyme activity of comparable magnitude to the increases in cyt. P-450 isozyme content.

If it is accepted that the probable explanation of our results is that certain phenoxazone ethers show high and differing substrate selectivities for different induced isozymes of cyt. P-450, then the next question must be to ask which isozymes show selectivity for which ethers. The consensus of existing literature on other substrates suggests that it is unlikely that any one phenoxazone ether will be specific for only one isozyme of cyt. P-450. We have shown here that one PB-induced isozyme and one BNF- (or MC-)-induced isozyme (probably the major induced isozyme in each instance) display widely differing selectivities for the phenoxazone ethers, but PB and MC are each known to induce more than one isozyme. Since MC and ISO induce a pair of isozymes in common, namely P-450c (probably identical with P-450$_{BNF-B2}$ and also known as P-450$_{BNF-B}$) and P-450d (also called P-450$_{BNF/ISF-G}$) [2, 7], and since P-450c is the major isozyme induced by MC whereas P-450d is the major isozyme induced by ISO, then our observation that ISO preferentially induced the metabolism of benzyloxyphenoxazone rather than that of the highly P-450c-selective substrate, ethoxyphenoxazone, suggests that in ISO-induced microsomes the benzyl ether was highly selective for P-450d. The large induction of the microsomal metabolism of benzyloxyphenoxazone by PB is consistent with our finding that this is a preferred substrate also of the major PB-induced cyt. P-450 isozyme. The very large induction by SKF-525A of the microsomal metabolism of both the pentyl and benzyl ethers raises the question of whether this was due to the induction of the same isozyme that is induced as the major isozyme by PB, or whether these substrates were showing selectivity for an entirely different SKF-525A-induced isozyme. Although PB and PCN each induce the isozyme P-450$_{PB/PCN-E}$ (which is not the major PB-induced isozyme) [2], our observation that PCN caused very little induction of the metabolism of any phenoxazone ether suggests that the pentyl and benzyl ethers, whose metabolism was induced greatly by PB, were not selective substrates for P-450$_{PB/PCN-E}$. We plan to answer the above questions directly using purified (ISO-, SKF-, PB- and PCN-)induced cyt. P-450 isozymes.

A very important question concerning specificity is whether or not in the microsomal membrane the substrate specificity of one cyt. P-450 isozyme will be affected by the immediate presence of other isozymes. A recent report concerning reconstituted monooxygenase systems containing more than one cyt. P-450 isozyme indicates that there is indeed selective modulation of the activity of one isozyme by another [20]. This may provide an explanation for our results in ARO-induced rats: based on the rates of metabolism of ethoxy-, pentoxy- and benzyloxyphenoxazone we observed that, in comparison to MC (or BNF) and PB, ARO caused maximal "MC-like" induction of dealkylase activity but only fractional "PB-like" dealkylase induction, yet reports of immunoquantitation studies have shown that ARO induces as large an increase in the microsomal amounts of each of the major PB- and MC-inducible cyt. P-450 isozymes as do PB and MC (or BNF) themselves [3, 4, 7, 25]. We are currently investigating whether concomitant induction of the major PB- and MC-inducible isozymes result in the

apparent inhibition of the PB-inducible isozyme's phenoxazone dealkylase activity by the MC-inducible isozyme.

Finally, in addition to being valuable routine probes for cyt. P-450 induction, the phenoxazone ethers may be powerful tools with which to study the enzymic factors that govern the substrate specificities of cyt. P-450. Why, for example, does a change of just three carbons in the side-chain length (from ethoxy to pentoxy) result in a radical change in isozyme specificity? We have provided evidence elsewhere that the differences in specificity for the homologous alkoxyphenoxazones between different induced cytochromes P-450 may involve differences in the transformation of enzyme-bound substrate (e.g. differences in transition states), rather than differences in the binding of substrate to cyt. P-450 [9].

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REFERENCES

1. R. Snyder and H. Remmer, in *Hepatic Cytochrome P-450 Monooxygenase System* (Eds. J. B. Schenkman and D. Kupfer), pp. 227–268. Pergamon Press, New York (1984).
2. F. P. Guengerich, G. A. Dannan, S. T. Wright and M. V. Martin, *Xenobiotica* **12**, 701 (1982).
3. F. P. Guengerich, G. A. Dannan, S. T. Wright, M. V. Martin and L. S. Kaminsky, *Biochemistry* **21**, 6019 (1982).
4. S. L. Seidel, L. K. Shawver and T. K. Shires, *Archs Biochem. Biophys.* **229**, 519 (1984).
5. L. S. Kaminsky, F. P. Guengerich, G. A. Dannan and S. D. Aust, *Archs Biochem. Biophys.* **225**, 398 (1983).
6. T. Wolff, E. Deml and H. Wanders, *Drug Metab. Dispos.* **7**, 301 (1979).
7. D. E. Ryan, P. E. Thomas, L. M. Reik and W. Levin, *Xenobiotica* **12**, 727 (1982).
8. M. D. Burke and R. T. Mayer, *Drug Metab. Dispos.* **2**, 583 (1974).
9. M. D. Burke and R. T. Mayer, *Chem.-Biol. Interact.* **45**, 243 (1983).
10. M. D. Burke and R. T. Mayer, *Drug Metab. Dispos.* **3**, 245 (1975).
11. M. Falzon, A. S. Milton and M. D. Burke, *Biochem. Pharmac.* **33**, 1285 (1984).
12. T. Haaparanta, J. Halpert, H. Glaumann and J.-A. Gustafsson, *Cancer Res.* **43**, 5131 (1983).
13. M. K. Buening and M. R. Franklin, *Drug Metab. Dispos.* **4**, 244 (1976).
14. M. Dickins, C. R. Elcombe, S. J. Moloney, K. J. Netter and J. W. Bridges, *Biochem. Pharmac.* **28**, 231 (1979).
15. R. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
17. A. P. Alvares and A. Kappas, *J. biol. Chem.* **252**, 6373 (1977).
18. P. E. Thomas, L. M. Reik, D. E. Ryan and W. Levin, *J. biol. Chem.* **256**, 1044 (1981).
19. G. J. Fisher, H. Fukushima and J. L. Gaylor, *J. biol. Chem.* **256**, 4388 (1981).
20. M. Kitada, K. Sakamoto, T. Rikihisa and Y. Kanakubo, *Biochem. Pharmac.* **33**, 3971 (1984).
21. F. M. Goujon, D. W. Nebert and J. E. Gielen, *Molec. Pharmac.* **8**, 667 (1972).
22. P. E. Thomas, J. Reidy, L. M. Reik, D. E. Ryan, D. R. Koop and W. Levin, *Archs Biochem. Biophys.* **235**, 239 (1984).
23. W. R. Porter, C. Wheeler and W. F. Trager, *Biochem. Pharmac.* **30**, 3099 (1981).
24. D. Hultmark, K. Sundh, L. Johansson and E. Arrhenius, *Biochem. Pharmac.* **28**, 1587 (1979).
25. A. Parkinson, P. E. Thomas, D. E. Ryan, L. M. Reik, S. H. Safe, L. W. Robertson and W. Levin, *Archs Biochem. Biophys.* **225**, 203 (1983).