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Nonideal Behavior of Alkoxyphenoxazone Compounds (Cytochrome *P*-450 Substrates) in Aqueous Solution

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ABSTRACT: Spectral properties of the cytochrome *P*-450 substrates, methoxy-, ethoxy-, pentoxy-, and benzyloxyphenoxazone (MeOPx, EtOPx, PeOPx, and BzOPx, respectively) were investigated from 350 to 600 nm in ethanol and aqueous buffer. In ethanol, each alkoxyphenoxazone displayed a λ max at 460 nm and a shoulder around 390 nm. Extinction coefficients (E_{mM}) in ethanol were calculated as MeOPx, 20.5; EtOPx, 20.4; PeOPx, 24.7; and BzOPx, 22.4. In aqueous buffer, only MeOPx obeyed the Lambert-Beer law (λ_{max} = 480 nm, E_{mM} = 22.1). Three substrates, EtOPx, PeOPx, and BzOPx, displayed anomalous behavior in aqueous solution, wherein the λ_{max} shifted to lower wavelengths (480–430 nm) and E_{mM}(apparent) decreased as the alkoxyphenoxazone concentration increased. This behavior was dependent on the side chain, and the concentrations at which the spectral changes took place were estimated as: BzOPx, 2 μM; PeOPx, 5 μM; EtOPx, 17 μM; and MeOPx, > 20 μM. The blue shift and decreased E_{mM} (apparent) observed for PeOPx at high concentration in aqueous buffer was reversed at high temperature. Unlike EtOPx, PeOPx, and BzOPx, and like MeOPx, hydroxyphenoxazone (resorufin) and unsubstituted phenoxazone obeyed the Lambert-Beer law in aqueous buffer and ethanol. The data suggest that the pentoxy and benzyloxy substituents facilitated a self-association process among the phenoxazones in aqueous solution. The data further show that aqueous solutions should be avoided when spectral data are used to determine alkoxyphenoxazone concentrations.

KEY WORDS: **Alkoxyphenoxazone, Cytochrome *P*-450, Spectral Properties.**

INTRODUCTION

Alkoxyphenoxazone (ROPx) compounds are planar aromatic hydrocarbons that have found widespread use as substrates for cytochrome *P*-450-dependent enzymes (E.C. 1.14.14.1). The tricyclic phenoxazone structure is common to all ROPx's, and its area to depth ratio, which is similar to that of benzo[*a*]-pyrene, has been used to explain the preference of ethoxyphenoxazone (EtOPx) for the α-naphthoflavone (ANF)-sensitive P448 form (1). Other ROPx's, however, each with the same tricyclic ring structure, are substrates for the metyrapone (MP)-sensitive *P*-450 form (2–4). Hence the alkoxy side-chain confers properties on the ROPx compounds that permit them to distinguish between different cytochrome *P*-450-dependent enzymes.

During our studies on pulmonary alkoxyphenoxazone dealkylase (ROPxase) activities, we recognized the saturation region for one substrate, EtOPx, was narrow (3, 4) and decided to use extinction coefficients (E_{mM}'s) to determine the concentrations of substrate solutions accurately. Because the literature was inconsistent (2, 5), we set out to determine the E_{mM} for four commercially available ROPx's but found that not all of them obeyed the Lambert-Beer law (6) in aqueous solutions. Furthermore, the nonideal behavior was most distinct for substrates for the MP-sensitive *P*-450 form. We felt it was necessary to study this behavior of the four ROPx's for two reasons. Incorrect extinction coefficients could have consequences for the interpretation of experimental data [e.g., if the saturation region is small (3, 4)]. In addition, a knowledge of the solution behavior of the ROPx's should suggest additional biochemical studies designed to investigate how the alkoxy side chain influences the interaction between a phenoxazone compound and a *P*-448 or a *P*-450 form. Such information might be useful for designing toxicologi-

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cal studies, where the forms responsible for catalyzing the *O*-dealkylation of ROPx's are differentially sensitive to the presence of environmental contaminants (7).

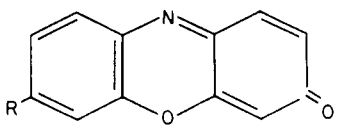
In this communication, we describe the spectral properties of methoxyphenoxazone (MeOPx), ethoxyphenoxazone (EtOPx), pentoxyphenoxazone (PeOPx), and benzyloxyphenoxazone (BzOPx) in organic and aqueous solutions. Where the Beer-Lambert law was obeyed, E_{mM}'s are given. The effects of solute concentration and temperature on the nonideal behavior in aqueous solution are also presented.

METHODS

All compounds were commercially available (Molecular Probes, Eugene, OR) and judged pure by TLC. We studies the alkoxy derivatives, MeOPx, EtOPx, PeOPx, and BzOPx as well as hydroxyphenoxazone (resorufin) and the unsubstituted phenaxazone. The structures are represented in Figure 1.

Stock solutions were prepared in dimethylsulfoxide (DMSO), the solvent we found to be the most efficient for all the compounds. The concentration of each stock solution was based on weight and depended on solubility. In the case of the ROPx's, concentrations of stock solutions ranged from 0.4 mM for MeOPx to 2 mM for PeOPx.

Absorbance spectra were recorded on a Model 17D Cary recording spectrophotometer at ambient temperature (22-26°C), except where indicated. When temperature dependence studies were carried out, water was circulated through jacketed cell holders from a circulating water bath. Samples, at an appropriate concentration were incubated for 15 min in the water bath under dimmed lights to prevent photodecomposition, and the cuvettes were kept in the cell holders during that time.



R = H Phenoxazone
R = HO Hydroxyphenoxazone (Resorufin)
R = CH_3O Methoxyphenoxazone
R = C_2H_5O Ethoxyphenoxazone
R = $C_5H_{11}O$ Pentoxyphenoxazone

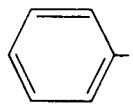


FIGURE 1. Structures of phenoxazone compounds.

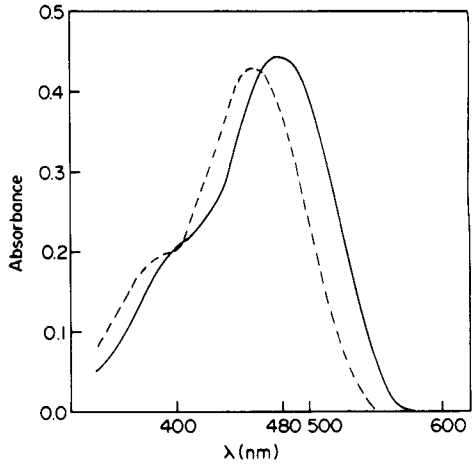


FIGURE 2. Spectra of Methoxyphenoxazone in ethanol and aqueous solution. (----), ethanol; ——, 0.05 M Hepes, pH 7.8; [MeOPx] = 19.6 μM.

Two solvents were used for the spectral studies: absolute ethanol (EtOH) and an aqueous solvent, 0.05 M HEPES, pH 7.8, hereafter called Hepes. Hepes was used for all experiments including the temperature-dependence studies, and EtOH was used at ambient temperature only. The final DMSO concentration was maintained at 2% (v/v) a DMSO concentration which itself did not induce spectral changes.

The expression E_{mM} represents an extinction coefficient (mM^{-1} cm^{-1}) calculated under conditions where the Lambert-Beer law was obeyed (6). The reported E_{mM} values are averages of duplicate experiments. When adherence to the Lambert-Beer law was not demonstrated, the term E_{mM}(apparent) is used.

RESULTS

In EtOH, the absorption spectrum of each alkoxyphenoxazone exhibited a peak at 460 nm and a shoulder at 390 nm. An example is given in Figure 2, which shows the visible spectrum of MeOPx. For each tested ROPx, the Lambert-Beer law was obeyed in EtOH. Extinction coefficients in EtOH were determined from duplicate experiments and they are given in Table 1. The change of solvent from EtOH to

TABLE 1. Extinction Coefficients in Ethanol for Four Alkoxyphenoxazones

ROPx	E (mM^{-1} cm^{-1}) (λ_{max} = 460 nm)	*Concentration range (μM)*
MeOPx	20.5	0.4-19.6
EtOPx	20.4	0.9-43.8
PeOPx	24.7	0.5-42.4
BzOPx	22.4	0.6-12.5

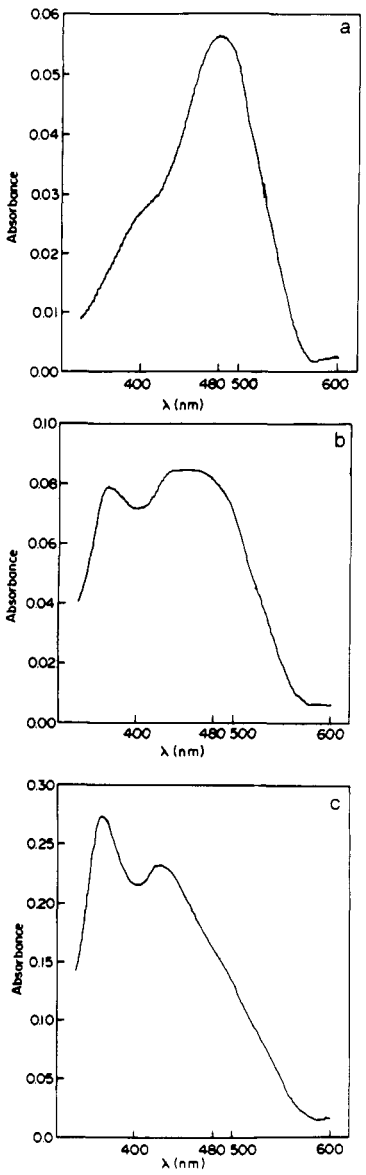


FIGURE 3. Spectra of pentoxyphenoxazone in aqueous solution (0.05 M Hepes, pH 7.8). (a) 2.12 μM, (b) 8.48 μM, (c) 42.4 μM.

Hepes had an affect on the spectrum of the MeOPx. As illustrated in Figure 2, the λ_{max} was red shifted by 20 nm. In the case of MeOPx, the Lambert-Beer law was obeyed from 0.4 to 19.6 μM and the E_{mM} at 480 nm was calculated as 22.1 in Hepes.

Anomalous behavior, however, was observed from the other ROPx's in Hepes. The λ_{max} changed over a range of wavelengths and the E_{mM}(apparent) decreased as the concentration increased. Data obtained for PeOPx in Hepes are shown in Figure 3. At low concentration (2.12 μM), the spectral properties of PeOPx were the same as those observed for MeOPx in Hepes. In contrast to MeOPx, however, two concentration-dependent changes took place as the concentration of PeOPx increased. The high wavelength peak shifted to the blue, where it became a broad peak around 440 nm at 8.48 μM and then a sharp but minor peak at 430 nm at 42.4 μM. The shoulder at about 390 nm at 2.12 μM, changed to a sharp major peak at 370 nm at 42.4 μM PeOPx.

The concentration-dependent changes described for PeOPx in Hepes were also observed for EtOPx and BzOPx. The sidechain, however, played a role in the extent of the spectral shifts. The blue shift observed for PeOPx occurred for EtOPx at a higher concentration, whereas the same changes occurred for BzOPx at lower concentrations. Quantitatively, the decrease in E_{mM}(apparent) that accompanied the blue shift for PeOPx also occurred for EtOPx and BzOPx. The Hepes-induced spectral changes are shown graphically in Figure 4, a-d, where E_{mM}(apparent) for the peak at $\lambda_{max} > 400$ nm is presented. For PeOPx and BzOPx, the low or limiting value of E_{mM}(apparent), at λ_{max}, is associated with a peak at 430 nm. Because the spectral changes for EtOPx do not occur until relatively higher concentrations, the peak at 430 nm was not present. The ROPx concentration at which the spectral changes occurred may be estimated from Figure 4, a-d, as: BzOPx, 2 μM; PeOPx, 5μM; and EtOPx, 17 μM. No spectral changes were observed for MeOPx up to 20 μM.

At $\lambda < 400$ nm, the shallow shoulder at about 390 nm for low ROPx concentration also experienced a blue-shift and at high PeOPx and BzOPx concentrations became a sharp, major peak. While the decrease in absorbance and the blue shift in Hepes were observable over wide PeOPx and BzOPx concentrations, the presence of the discrete peaks at 370 nm and 430 nm occurred only at the higher concentrations. Beyond the concentrations shown in Figures 4, turbidity was observed, and the PeOPx and BzOPx concentration points available for calculating E_M's at 370 nm and 430 nm were limited. To overcome this difficulty, data were combined from duplicate experiments and the E_{mM}'s were calculated as: PeOPx, 5.0 (430 nm) and 6.5 (370 nm); BzOPx, 6.5 (430 nm) and 9.6 (370 nm).

To determine the extent to which the phenoxazone ring structure or a highly polar substituent affected the spectral properties, we studied phenoxazone and hydroxyphenoxazone (resorufin) in EtOH and Hepes. The visible spectrum for unsubstituted phenoxazone showed two peaks in EtOH (350 nm, 445 nm), each of which experienced a red shift (360 nm, 460 nm) in the aqueous Hepes buffer. Each λ_{max} was constant with concentration, and absorbance followed the Lambert-Beer law. Calculated E_{mM}'s are in Table 2.

The spectra of resorufin in EtOH and Hepes were different from phenoxazone and the ROPx, although absorbance was linearly related to concentration. In EtOH a major peak occured at 578 nm with two shoulders at 560 nm and 542 nm. The major peak was blue-shifted in aqueous buffer to 570 nm and the

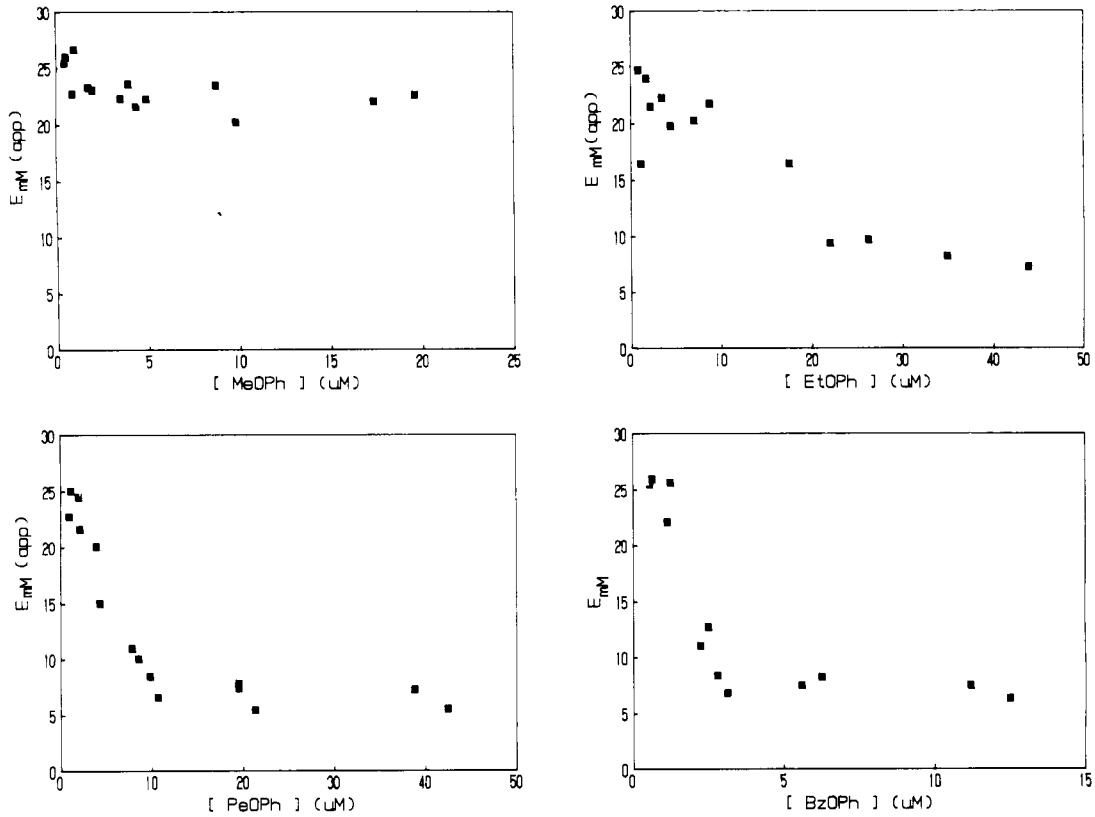


FIGURE 4. Effect of varying alkoxyphenoxazone concentration on E_{mM}(apparent) in 0.05 M Hepes, pH 7.8.

shoulders were less distinct. Calculated E_{mM}'s are in Table 2.

To determine whether the anomalous behavior in aqueous buffer was temperature sensitive, PeOPx spectra were recorded at 8–70° C. The temperature dependence was observed at 2.12 μM, 8.48 μM, and 42.4 μM PeOPx, the concentrations used for the spectra recorded at ambient temperature (Figure 5). The λ_{max} (480 nm) and absorbance for 2.12 μM PeOPx in the aqueous Hepes remained constant from 8 to 70° C (data not shown). The spectra for 8.48 μM and 42.4 μM PeOPx were temperature sensitive, and the profile was distinct for each concentration. The data in Figure 5a show that for 8.48 μM PeOPx, the 430-nm and 370-nm peaks occured at 8° C, whereas the 480-nm peak appeared by 60° C. The spectra for 42.4 μM PeOPx (Figure 5b), however, exhibited only a partial reversal at temperatures as high as 70° C.

TABLE 2. Extinction Coefficients for Phenoxazone and Resorufin in Ethanol and Hepes Buffer

Compound	E (mM^{-1} cm^{-1}) EtOH	Aqueous buffer
Phenoxazone	10.2 (445)[a]	9.6 (460)
	12.6 (350)	11.5 (360)
Resorufin	93.8 (578)	76.5 (570)

[a] Numbers in parentheses represent the λ_{max}.

DISCUSSION

The spectral studies described herein focus on the visible region of the spectrum. The 460-nm peak probably represents the enone K band (6). The λ_{max} was red shifted compared to the unsubstituted phenoxazone, and the change from EtOH to aqueous Hepes buffer resulted in a red shift for MeOPx at all concentrations and for EtOPx, PeOPx, and BzOPx at low concentration.

Changes in spectral properties also took place in aqueous solution, and they were dependent on solute concentration. The extent of the concentration-dependent spectral changes followed the series BzOPx > PeOPx > EtOPx > MeOPx. The λ_{max} shifted to lower wavelengths and calculated E_{mM}(apparent)'s decreased as the ROPx concentration increased. Such behavior in aqueous solution is characteristic of systems where the absorbing species self-associate into discrete structures or nonspecific aggregates. Among classes of organic compounds known to self-associate are the fluoresciens, acridines, and simpler purines in which the association process is facilitated by the presence of bulky substituents (8–10).

Our experiments are based on the spectral properties of the phenoxazone ring and do not monitor the alkoxy substituents themselves. Other studies, how-

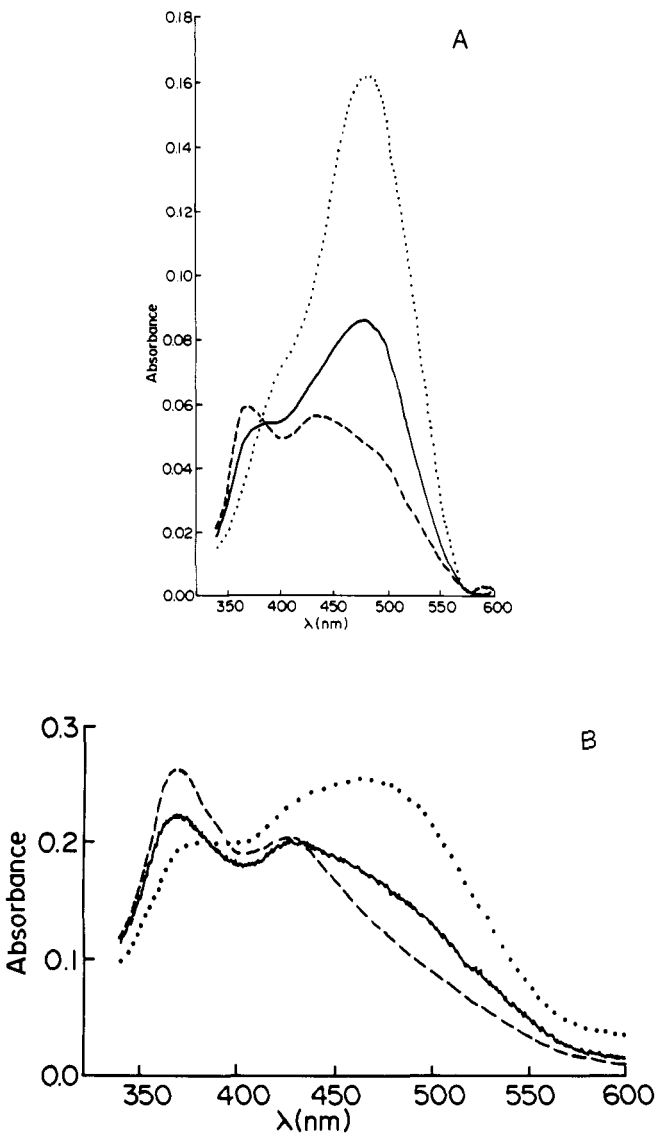


FIGURE 5. Effect of temperature on spectra of pentoxyphenoxazone at two concentrations in 0.05 M Hepes, pH 7.8. (A) 8.48 μM, (B) 42.4 μM; (-----) 8° C, ——, 34° C, (····) 61° C.

ever, have shown bulky alkyl substituents facilitate ring overlap (8, 11). Although the phenyl ring of BzOPx may itself be involved in a ring association, other methods (e.g., NMR) will be needed to monitor individual contributions.

The reversal of the PeOPx spectrum at high temperature is similar to that observed for purine compounds. In the latter case, the observations were explained in terms of base-stacking interactions which were reversed by the high temperatures (11, 12). The dependence on PeOPx concentration of the extent of the reversal at high temperature suggests that the solution structures of the complexes formed in Hepes at medium (8.5 μM) and high (42 μM) PeOPx concentrations were different. The structure of the complex formed at 42 μM is more stable to heat disruption than the structure formed at 8.5 μM PeOPx. Using adenine-aminoacridine dimers separated by methylene spacer arms, Constant *et al.* (13) also demonstrated that the form exhibiting the greatest overlap was the most resistant to heat disruption.

The spectral properties we have described are not only important from a theoretical perspective, they are important from a practical, experimental one. The ROPx's are commonly used in biochemical studies on cytochrome *P*-450-dependent metabolism, and concentrations are conveniently determined from spectral data. Such determinations take on more importance because of the narrow substrate saturation range of ROPx'ase activity (3, 4), and errors in concentration could result in nonvalid assays. Our data show that such determinations should be made in a nonaqueous system, and the appropriate E_{mM}'s for MeOPx, EtOPx, PeOPx, and BzOPx in EtOH are now available.

The E_{mM} we determined for resorufin is greater than that previously reported (5) and may be due to a more highly purified compound. Our previous studies were based on E_{mM} = 40.0 for resorufin (5), and the specific activities reported for the alkoxyphenoxazone dealkylases should be corrected by the factor 40.0/76.5 = 0.52.

The ROPx's used in this study are substrates for the microsomal membrane-bound cytochrome *P*-450-dependent monoxygenases. Furthermore, the PeOPx and BzOPx are specific for the MP-sensitive form, whereas MeOPx and EtOPx are substrates for the ANF-sensitive form (3). Although experiments carried out in bulk solution do not address the subtleties of membrane-mediated events, we want to suggest the nonideal aqueous solution behavior of some ROPx's may be important for an understanding of the interactions between *P*-488- and *P*-450-enzymes and the ROPx substrates. Membrane-mediated changes in the spectral properties of small molecules have been demonstrated (14–16). In the case of the ROPx's, the presence of the pentoxy- and benzyloxy sidechains permits these compounds to be substrates for a MP-sensitive *P*-450 form, despite the presence of the planar tricyclic phenoxazone moeity (3, 4). Future studies in which spectral changes are used to monitor interactions between phenoxazone compounds and synthetic or biological membranes should help us to gain additional insight into the role of the membrane in cytochrome *P*-450 catalytic activity.

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