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7-ALKOXYQUINOLINES: NEW FLUORESCENT SUBSTRATES FOR CYTOCHROME P450 MONOOXYGENASES

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Abstract—A series of 7-alkoxyquinolines was synthesized and tested as substrates with hepatic microsomes prepared from male Wistar rats. Microsomal O-dealkylation rates and kinetic constants were determined for the 7-alkoxyquinolines with microsomes from control, 3-methylcholanthrene (MC)-pretreated, and phenobarbitone (PB)-pretreated rats. Structure–activity relationship studies indicated that the 7-benzyloxyquinoline was the most rapidly metabolized substrate for control microsomes and those from PB-pretreated rats, whereas the 7-ethoxy- and 7-propoxyquinolines were O-dealkylated more rapidly by microsomes of MC-pretreated animals. Differences in activities occurred in V_{max} and apparent K_m values; however, there does not appear to be a correlation between these two values for the different quinoline substrates. Apparent K_m and V_{max} values for the 7-alkoxyquinolines were: control microsomes, K_m = 71–773 μM, V_{max} = 0.37–8.4 nmol 7-quinolinol/min/mg protein; MC microsomes, K_m = 0.5–14 μM, V_{max} = 0.29–2.7 nmol 7-quinolinol/min/mg protein; PB microsomes, K_m = 2.8–46 μM, V_{max} = 0.9–12 nmol 7-quinolinol/min/mg protein. All of the quinoline substrates gave Type I binding spectra with control and MC microsomes. With PB microsomes, Type I, Reverse Type I, and a mixture of the two types of binding spectra were observed. Comparisons of the structure–activity relationships, levels of induction, and kinetic constants were made with 7-alkoxycoumarin and 7-alkoxyphenoxazone analogs. In addition, three new coumarin substrates (7-pentoxy-, 7-hexoxy-, and 7-benzyloxycoumarin) are described.

Several reports attest to the fact that there are multiple forms of mammalian microsomal cytochromes P450 [1–4]. The different forms may have different, similar, or overlapping substrate specificities, making it difficult to separate and/or determine the resident activities of particular cytochromes P450 in cells, tissue sections or microsomal preparations. Factors influencing substrate specificity include such things as the innate character of the cytochrome, e.g. the hydrophobicity of the cytochrome and the regio- and stereospecificity of the active site [1, 5]. Progress has been made in understanding substrate specificity via determination of the primary, secondary and tertiary structures of various forms of cytochrome P450 [2, 3, 6, 7] and the use of site-directed mutagenesis [8].

Another approach or aid in understanding substrate specificity is that of using substrates to determine enzyme active site characteristics. Substrates have been synthesized that appear to fit particular cytochrome P450 active sites based on the high degree of substrate specificity. Examples would be those of 7-ethoxy- and 7-pentoxyphenoxazones (resorufins) which are, respectively, highly specific substrates for the 3-methylcholanthrene (MC) and phenobarbitone (PB) inducible forms of cytochrome P450 [9–11]. These substrates have a distinct advantage over other less selective substrates when it comes to studying variant forms of cytochrome P450 in heterogenous mixtures because the metabolic activities observed are those of the PB and MC variant forms; other cytochromes P450 are minimally active with these substrates [9–11].

We have had a continuing interest in developing new, fluorescent substrates for cytochrome P450 monooxygenases in an effort to expand our knowledge of these enzymes as well as providing sensitive and easily used assays. In addition, we have sought to develop isoenzyme-specific substrates with good metabolic activities (i.e. high turnover numbers) to ensure accurate measurements. Ideally, the rates of metabolism for the substrates would approach those of physiological substrates. Recently we introduced 7-methoxyquinoline for the assay of hepatic microsomal cytochrome P450 monooxygenases [12]. One of the purposes in developing the 7-alkoxyquinoline substrates was to determine if the nitrogen in the ring contributed metabolic specificity similar to that observed with the phenoxazone substrates. The phenoxazones are the only fluorescent substrates available that have both nitrogen and oxygen atoms in the aromatic rings. While the 7-alkoxycoumarins are more highly metabolized by PB- and MC-induced forms of cytochrome P450, they lack the specificity of the phenoxazone substrates [13–19].

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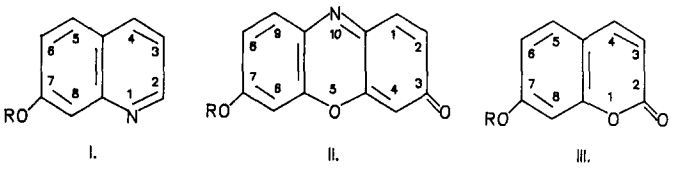

I. II. III.

R = -H (7-Hydroxy-)
= $-CH_3$ (7-Methoxy-)
= $-CH_2CH_3$ (7-Ethoxy-)
= $-CH_2CH_2CH_3$ (7-Propoxy-)
= $-CH_2(CH_2)_2CH_3$ (7-Butoxy-)
= $-CH_2(CH_2)_3CH_3$ (7-Pentoxy-)
= $-CH_2(CH_2)_4CH_3$ (7-Hexoxy-)
= $-CH_2(C_6H_5)$ (7-Benzyloxy-)

Fig. 1. Structures of 7-substituted quinoline (I), phenoxazone (II), and coumarin (III) substrates and products.

We have now prepared a series of 7-alkoxyquinolines (7-methoxy- to 7-hexoxy- and 7-benzyloxyquinoline; Fig. 1, structure I) and determined their metabolic activities with control, MC-, and PB-induced rat hepatic microsomal cytochrome P450 monooxygenases. Comparisons are made with similar 7-alkoxyphenoxazone and 7-alkoxycoumarin substrates (see Fig. 1, structures II and III).

MATERIALS AND METHODS

Chemicals. $NADP^+$, NADPH, glucose-6-phosphate (G6P), and glucose-6-phosphate dehydrogenase (G6PD) were purchased from Boehringer Mannheim* (Mannheim, F.R.G.). Phenobarbital and Tris were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). 3-Methylcholanthrene was obtained from Fluka AG (Buchs, Switzerland). 7-Quinolinol (7-hydroxyquinoline), 7-hydroxyphenoxazone (resorufin), and 7-hydroxycoumarin were purchased from Kodak Laboratory Chemicals (Rochester, NY, U.S.A.). The alkyliodides were from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All other chemicals were of reagent quality or the highest quality available.

Animals and sample preparation. Male Wistar rats (approximately 200 g each) were obtained from a colony maintained at the Institute for Pharmacology and Toxicology, Marburg, FRG. Animals were given various chemical pretreatments as follows. PB was administered in the drinking water (0.1%, w/v, for 6 days) and MC was injected (once i.p., 30 mg/kg body wt) as a peanut oil solution. Controls were injected once with 0.5 to 1.0 mL of peanut oil. No difference in activities were observed between control animals receiving water or injections of peanut oil. Animals were killed by cervical dislocation 2 days after the last MC injection or after 6 days of oral PB administration. Food was removed the night before the animals were killed. Livers were removed, weighed, cut into small pieces, and rinsed several times with ice-cold 20 mM Tris–HCl (pH 7.6) containing 150 mM KCl and 1 mM EDTA. The rinse solution was removed and the livers were homogenized in 4 vol. of the same buffer. All operations were conducted at 4°. Liver homogenates were then centrifuged at 1,000 *g* for 10 min and then the supernatant fractions were transferred to clean centrifuge tubes and centrifuged at 10,000 *g* for 10 min. The 10,000 *g* supernatant fractions were removed and centrifuged at 100,000 *g* for 60 min. The pellets were resuspended in 20 mM Tris–HCl (pH 7.6), containing 150 mM KCl and 3 mM $MgCl_2$ and recentrifuged at 100,000 *g* for 60 min. Microsomal pellets obtained in this fashion were finally suspended in 20 mM Tris–HCl (pH 7.6) containing 150 mM KCl, 3 mM $MgCl_2$ and 15% glycerin using a ratio of 1 mL buffer/g of fresh liver.

Protein concentrations were determined by the method of Lowry *et al.* [20]. Cytochrome P450 measurements followed the procedure of Omura and Sato [21].

Synthesis of 7-alkoxyquinolines, 7-alkoxycoumarins, and 7-alkoxyphenoxazones. 7-Alkoxyquinolines were synthesized by previously published methods [12]. The 7-alkoxycoumarins were prepared according to Prough *et al.* [22]. The 7-alkoxyphenoxazones have been prepared and reported previously from this laboratory [10, 22, 23]. Mass spectra were obtained using a Hewlett–Packard (Sunnyvale, CA, U.S.A.) MSD 5970B mass spectrometer interfaced with a Hewlett–Packard GLC 5890 equipped with a 10 m OV-1 capillary column (Altech, Deerfield, IL, U.S.A.). Analytical conditions were: column flow rate, 1 mL helium/min; linear temperature program of 80–300° over 50 min; 2 psi head pressure; split was 40:1. Proton NMR spectra were obtained with a Varian Instruments Gemini 200 (Palo Alto, CA, U.S.A.) NMR spectrometer. R_f values for the quinolines were obtained using TLC plates (0.25 mm thick silica gel, 20 × 20 cm, Merck, Darmstadt, FRG) developed with $CHCl_3$:acetone (80:20). R_f values for 7-alkoxycoumarins were developed in a similar manner but with a benzene:ethanol:acetic acid (96.5:3:0.5) solvent system. Mass and proton NMR spectral correlations, melting points (uncorrected), and R_f values for new compounds are as follows:

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7-Methoxyquinoline—NMR (CCl_4/TMS) —$\underline{CH_3}$ = 4.1 ppm, singlet; aromatic protons (6H) = 7.15–9.1 ppm, multiplet; other data given previously [12].

7-Ethoxyquinoline—NMR ($CDCl_3$/TMS) —$\underline{CH_3}$ = 1.16–1.6 ppm, triplet; —$\underline{CH_2}$— = 4.0–4.55 ppm, quartet; aromatic protons (6H) = 7.1–9.1 ppm, multiplet. Mass spectral fragmentation pattern: M^+ at 173 (59%); M^+-C_2H_4 at 145 (100%); M^+-C_3H_4O at 117 (26%). Product was a light brown oil. R_f = 0.37.

7-Propoxyquinoline—NMR (CCl_4/TMS)—$\underline{CH_3}$ = 0.8–1.25 ppm, triplet; $CH_3\underline{CH_2}CH_2$— = 1.46–2.14 ppm, sextet; $CH_3CH_2\underline{CH_2}$— = 3.78–4.13 ppm, triplet; aromatic (6H) = 6.92–8.94, multiplet. Mass spectral fragmentation pattern: M^+ at 187 (31%) M^+-C_3H_6 at 145 (100%); M^+-C_4H_6O at 117 (18%). Product was a light brown oil. R_f = 0.40.

7-Butoxyquinoline—NMR ($CDCl_3$/TMS) $\underline{CH_3}$ $(\underline{CH_2})_2CH_2$— = 0.7–2.1 ppm, $CH_3(CH_2)_2\underline{CH_2}$— = 3.94–4.32 ppm, triplet; aromatic (6H) = 7.08–9.22 ppm, multiplet. Mass spectral fragmentation pattern: M^+ at 201 (24%); M^+-C_4H_8 at 145 (100%); M^+-C_5H_8O at 117 (14%). Product was a light brown oil. R_f = 0.44.

7-Pentoxyquinoline—NMR ($CDCl_3$/TMS) $\underline{CH_3}$ $(\underline{CH_2})_3$ CH_2— = 0.74–2.22 ppm, multiplet; $CH_3(CH_2)_3$ $\underline{CH_2}$– = 4.0–4.39 ppm, triplet; aromatic (6H) = 7.08–9.1 ppm, multiplet. Mass fragmentation pattern: M^+ at 215 (18%); M^+-C_5H_{10} at 145 (100%); M^+-$C_6H_{10}O$ at 117 (17%). Product was tan crystalline material, m.p. = 35–35.6°. R_f = 0.47.

7-Hexoxyquinoline—NMR ($CDCl_3$/TMS) $\underline{CH_3}$ $(\underline{CH_2})_4CH_2$— = 0.68–2.1 ppm, multiplet; $CH_3(CH_2)_4$ $\underline{CH_2}$— = 3.9–4.24 ppm, triplet; aromatic (6H) = 7.04–8.98 ppm, multiplet. Mass fragmentation pattern: M^+ at 229 (15%); M^+-C_6H_{12} at 145 (100%); M^+-$C_7H_{12}O$ at 117 (14%). Product was light tan crystalline material; m.p. = 35–35.6°. R_f = 0.48.

7-Benzyloxyquinoline—NMR ($CDCl_3$/TMS) —$\underline{CH_2}$— = 5.13–5.22 ppm, singlet; aromatic (11H) = 7.0–9.0 ppm, multiplet. Mass fragmentation pattern: M^+ at 235 (31%); M^+-C_9H_6NO at 91 ($C_7H_7^+$, 100%). Product was a light tan crystalline material; m.p. = 72.2–73.2°. R_f = 0.44.

7-Pentoxycoumarin—NMR (CCl_4/TMS) $\underline{CH_3}$ $(\underline{CH_2})_3CH_2$— = 0.7–2.02 ppm, multiplet; $CH_3(CH_2)_3$ $\underline{CH_2}$— = 3.82–4.12 ppm, triplet; aromatic (5H) = 5.98–7.7 ppm, multiplet. Mass fragmentation pattern: M^+ at 232 (39%); M^+-C_5H_{10} at 162 (100%); M^+-$C_6H_{10}O$ at 134 (79%). Product was a golden oil. R_f = 0.37.

7-Hexoxycoumarin—NMR (CCl_4/TMS) $\underline{CH_3}$ $(\underline{CH_2})_4CH_2$— = 0.65–2.08 ppm, multiplet; $CH_3(CH_2)_4$ $\underline{CH_2}$— = 3.74–4.15 ppm, triplet; aromatic (5H) = 6.0–7.82 ppm, multiplet. Mass fragmentation pattern: M^+ at 246 (37%); M^+-C_6H_{12} at 162 (100%); M^+-$C_7H_{12}O$ at 134 (69%). Product was a golden oil. R_f = 0.38.

7-Benzyloxycoumarin—NMR ($CDCl_3$/TMS) $-\underline{CH_2}$— = 5.1–5.28 ppm, singlet; aromatic (10H) = 6.16–7.80 ppm, multiplet. Mass fragmentation pattern: M^+ at 252 (6%); M^+-$C_9H_5O_3$ at 91 ($C_7H_7^+$, 100%). Light tan, monoclinic crystals; m.p. = 156.8–158.7°. R_f = 0.35.

Fluorescence O-dealkylation assays. Fluorescence of the products 7-hydroxycoumarin, resorufin, and 7-quinolinol was measured using a Perkin–Elmer (Norwalk, CT, U.S.A.) model 204-A spectrofluorometer. The reaction buffer for all reactions was 20 mM Tris (pH 7.6), 3 mM $MgCl_2$ and 150 mM KCl. All reactions were conducted at 25°. Substrates were prepared in dimethylsulfoxide (DMSO) as 1–20 mM stocks and added to the reactions with microliter syringes.

7-Alkoxyquinoline *O*-dealkylases were measured according to Mayer *et al.* [12] at excitation and emission wavelengths of 410 and 510 nm respectively.

7-Alkoxyphenoxazone *O*-dealkylase activities were determined by the procedure of Burke and Mayer [10], except that NADPH (250 μM final concentration), instead of an NADPH-generating system, was added directly to initiate the reaction. Microsomal protein for the 7-alkoxyphenoxazone *O*-dealkyase reactions ranged from 10 to 100 μg/mL. 7-Alkoxyphenoxazone concentrations in the reaction mixtures were from 20 to 450 nM. Resorufin was measured at excitation and emission wavelengths of 530 and 585 nm respectively.

7-Alkoxycoumarin *O*-dealkylase activities were determined by directly monitoring the production of 7-hydroxycoumarin in the reaction cuvettes. 7-Alkoxycoumarin concentrations in the reactions varied from 20 to 300 μM (final concentration). An NADPH-generating system was utilized to maintain constant levels of NADPH. The generation system (154 nmol G6P, 3–5 units G6PD and 100 nmol $NADP^+$) was added to the reaction cuvette which contained 2 mL (final volume) of reaction buffer and 200 μg of microsomal protein per mL. The mixtures were allowed to preincubate for 90 sec to allow for formation of NADPH before substrate was added to initiate the *O*-dealkylase reaction. 7-Hydroxycoumarin production was monitored at excitation and emission wavelengths of 390 and 440 nm respectively. Known amounts of 7-hydroxycoumarin were added to the reaction cuvettes, and the fluorescence was recorded to establish a calibration curve.

Cytochrome P450–7-alkoxyquinoline binding spectra. The binding of 7-alkoxyquinolines to oxidized microsomal cytochrome P450 suspensions was determined by the method of Schenkman [24]. An Aminco (SLM-Aminco, Urbana, IL, U.S.A.) DW-2 UV-Vis spectrophotometer in the split beam mode was employed to record spectra between 350 and 470 nm. Artifacts due to absorption of light by the 7-alkoxyquinolines in this wavelength region were eliminated by use of tandem cuvettes [10].

pH *Titration of 7-quinolinol* The effects of pH on the fluorescence of 220 μM 7-quinolinol solutions were determined over a pH range of 3 to 13. The solutions were made up in 50 mM citrate-KOH. Fluorescence was recorded at 515 nm using excitation wavelengths of 350, 362, and 404 nm. Fluorescence was recorded using an SLM-Aminco 500C spectrofluorometer (SLM-Aminco).

RESULTS

pH *Effects on 7-quinolinol fluorescence.* It has long

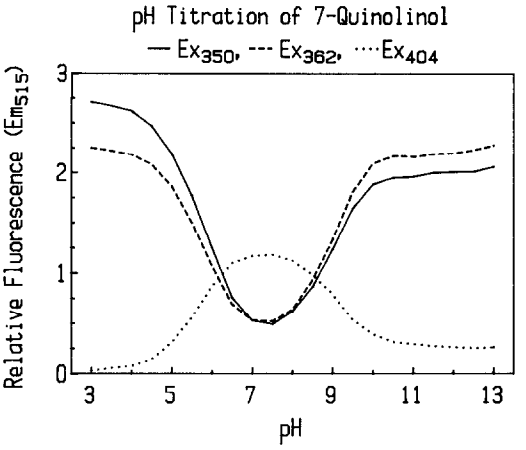

Fig. 2. pH Dependence of the fluorescence of 7-quinolinol. Fluorescence was measured at 515 nm using the three long-wave excitation maxima for acid (Ex_{350}), alkaline (Ex_{362}), and neutral (Ex_{404}) environments. Other conditions are given in Materials and Methods.

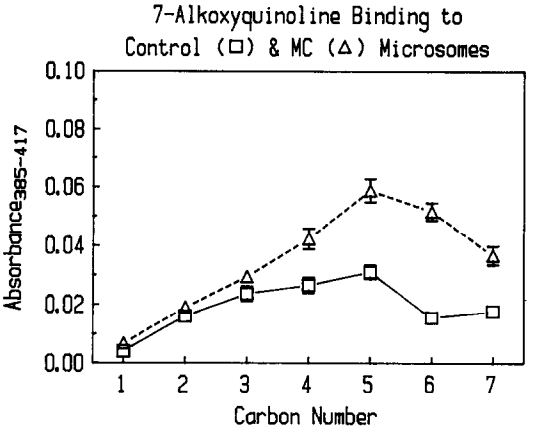

Fig. 3. 7-Alkoxyquinoline Type I binding to control and MC hepatic microsomes. Concentrations of the quinolines were 80 μM and microsomal suspensions were 2 mg/mL. Values are means ± SD; determinations were made from three different preparations of each of the treatment groups. Cytochrome P450 concentrations were as follows: controls = 0.61–0.97 nmol/mg protein; MC = 0.93–1.25 nmol/mg protein. Other conditions are given in Materials and Methods. The numbers along the abscissa indicate the length and type of the sidechain. Number 7 indicates the 7-benzyloxyquinoline.

been known that the observed fluorescence of a molecule with ionizable groups is often affected by the pH of the solution that the molecule is in [25]. Changes in the excitation and emission spectra of 7-quinolinol coincide with changes in the ionic species; there are four species (neutral, anion, cation, and zwitterion) of which two or more may be present in an aqueous environment [26]. Long wavelength excitation maxima at pH values of 4, 7, and 10 were, respectively, 350, 404, and 362 nm. Smaller differences were recorded in the emission maxima with the largest shift being no more than 5 nm, i.e. 512–517 nm using uncorrected spectra.

The fluorescence of 7-quinolinol was determined over a pH range of 3 to 13 (Fig. 2). Fluorescence was maximal from pH 7 to 8 and fell off sharply below pH7.0 and above pH8.0 when excited at 404 nm. The titration curves obtained using excitation maxima of 350 and 362 nm paralleled each other and were almost mirror images of that obtained at an excitation of 404 nm. The upward swing of the Ex_{350} curve on the basic side of the titration curve is due to the fact that this wavelength is close to the excitation maximum (Ex_{362}) of the phenolate species of the molecule. The reverse would be true for the Ex_{362} curve. The fluorescence intensity at 505 nm approximately doubles in going from a neutral to acid (<pH 4) and basic (>pH 10) environments when excited at the appropriate long wavelength maxima.

Cytochrome P-450–Substrate spectral changes. Special interactions with various cytochrome P450 preparations with 7-methoxyquinoline have been reported previously [12]. Attempts were made to determine the type of spectral interaction and K_s (spectral binding constant [24, 27]) values for the other 7-alkoxyquinolines with control, MC, and PB rat hepatic microsomes. All of the quinoline ethers gave very weak spectral interactions with control and MC microsomal suspensions. The difference spectra had absorbance maxima and minima at approximately 385 and 417 nm, respectively, which indicated Type I binding. Based on the magnitude of absorbance (i.e. $\Delta A_{385-417}$), 7-methoxyquinoline exhibited the weakest spectral interaction and 7-pentoxyquinoline the strongest for both control and MC microsomal preparations (Fig. 3). There were minimal differences in the ΔA values with 1C to 3C in the sidechains between control and MC microsomal preparations. The differences became greater with sidechains larger than 3C; the ΔA values for MC microsomes were 2-fold or greater than those of controls for the longer chain compounds (Fig. 3).

7-Alkoxyquinoline spectral interactions with PB microsomal preparations were more complex because Type I and Reverse Type I interactions were sometimes observed simultaneously in the difference spectra.

7-Methoxyquinoline exhibited exclusively Reverse Type I spectra regardless of concentration; absorbance maxima and minima were 416–417 and 390–392 nm respectively. The remaining compounds (7-ethoxy-, 7-propoxy-, 7-butoxy-, 7-pentoxy-, 7-hexoxy-, and 7-benzyloxyquinolines) produced either Type I, Reverse Type I, or a combination of the two. Manifestation of the spectra appeared to be concentration dependent. At 8 μM or less, only the Reverse Type I binding was observed (Figs. 4 and 5). Above 8 μM, either Type I or a combination of Type I and Reverse Type I binding was observed (Figs. 4 and 5). At 80 μM 7-pentoxy- and 7-hexoxyquinolines appeared to give only a Type I response; however, the Reverse Type I response may simply have been masked. The contribution of each type of binding (Type I = $\Delta A_{385-424}$, Rev. Type I = $\Delta A_{410-424}$) at 80 μM substrate concentrations can be seen in Fig. 6. The Reverse Type I component is only indicative of the extent of binding since the

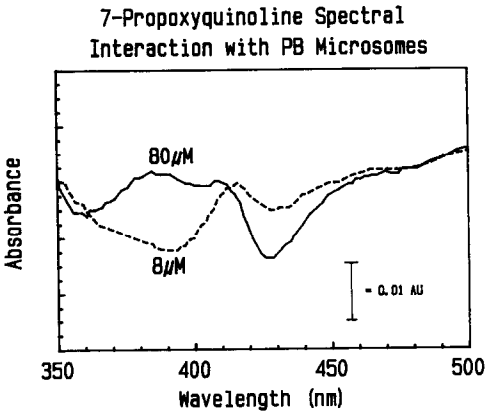

Fig. 4. 7-Propoxyquinoline spectral interactions with PB rat hepatic microsomes. Microsomal protein was 2 mg/mL suspension. Cytochrome P450 content was 2.27 nmol/mg protein. Binding spectra of 7-propoxyquinoline were recorded at 8 and 80 μM. Other conditions were as described in Materials and Methods.

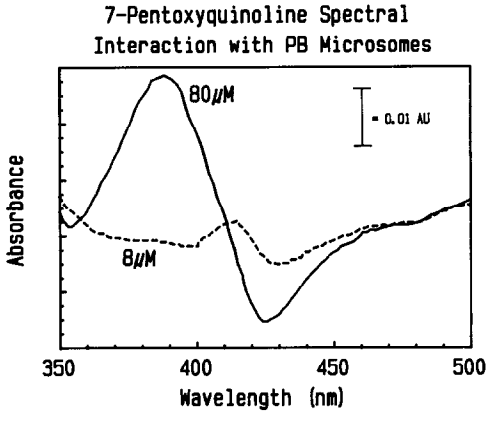

Fig. 5. 7-Pentoxyquinoline spectral interactions with PB rat hepatic microsomes. Conditions were as described in the legend of Fig. 4.

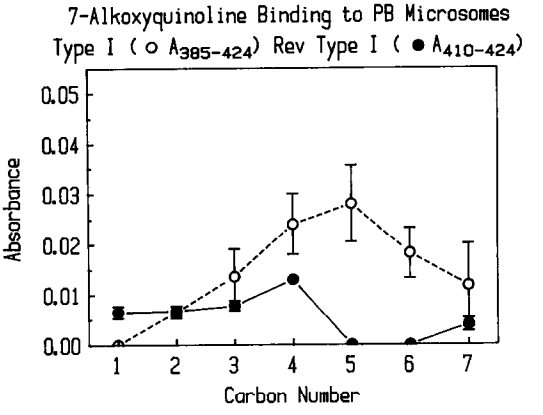

Fig. 6. Extent of Type I and Reverse Type I 7-alkoxyquinoline binding to PB microsomes. Conditions were as described in the legend of Fig. 3 except that the cytochrome P450 content was 1.69–1.94 nmol/mg protein. Values are means ± SD; determinations were made from three different preparations of each of the treatment groups. The extent of Type I binding was determined by measuring the absorbance difference between 385 and 424 nm, whereas Reverse Type I binding was determined between 410 and 424 nm. The numbers along the abscissa indicate the length and type of the sidechain. Carbon 7 indicates the 7-benzyloxyquinoline.

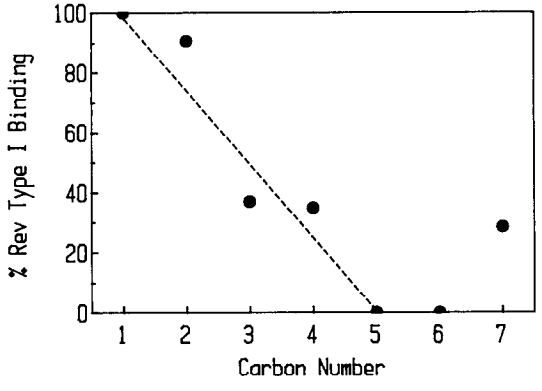

Fig. 7. 7-Alkoxyquinoline Reverse Type I binding as a percentage of total binding. Percent Reverse Type I binding was determined for each substrate as follows. % Reverse Type I = $[\Delta A_{410-424}/(\Delta A_{385-424} + \Delta A_{410-424})] \times 100$. The numbers along the abscissa indicate the length and type of the sidechain. Carbon 7 indicates the 7-benzyloxyquinoline.

wavelengths used were not at the exact absorbance maxima and minima. An estimate of the percentage of the Reverse Type I component for 80 μM concentrations of quinolines was made using the ΔA values for both types of binding (Fig. 7). There is an apparent linear relationship between the percentage of Reverse Type I interaction and the length of the sidechain between 7-methoxy- and 7-pentoxyquinoline.

Hexobarbital and metyrapone, classic Type I and Type II binding compounds, were tested to determine if they would affect the spectral interaction of 7-propoxyquinoline with PB microsomal suspensions. When hexobarbital was present (200 μM final concentration), no spectral perturbations were observed with 80 μM 7-propoxyquinoline, i.e. hexobarbital blocked binding. Metyrapone at 30 μM had no effect on the binding of 8–80 μM 7-propoxyquinoline.

Structure–activitiy relationships and induction of fluorescent substrate metabolism in hepatic microsomes. Structure–activity relationships (SARs) for the 7-alkoxyquinolines, -coumarins, and -phenoxazones are given in Fig. 8 A, B and C. The patterns of metabolic activity varied with the type of inducing agent and substrate used. Generally, metabolic activities in control microsomes were higher for the ethoxy, propoxy, butoxy and benzyloxy derivatives regardless of the type of ring system; this trend did not hold for induced microsomes.

7-Alkoxyquinoline metabolism in control microsomes increased in going from the methoxy to the

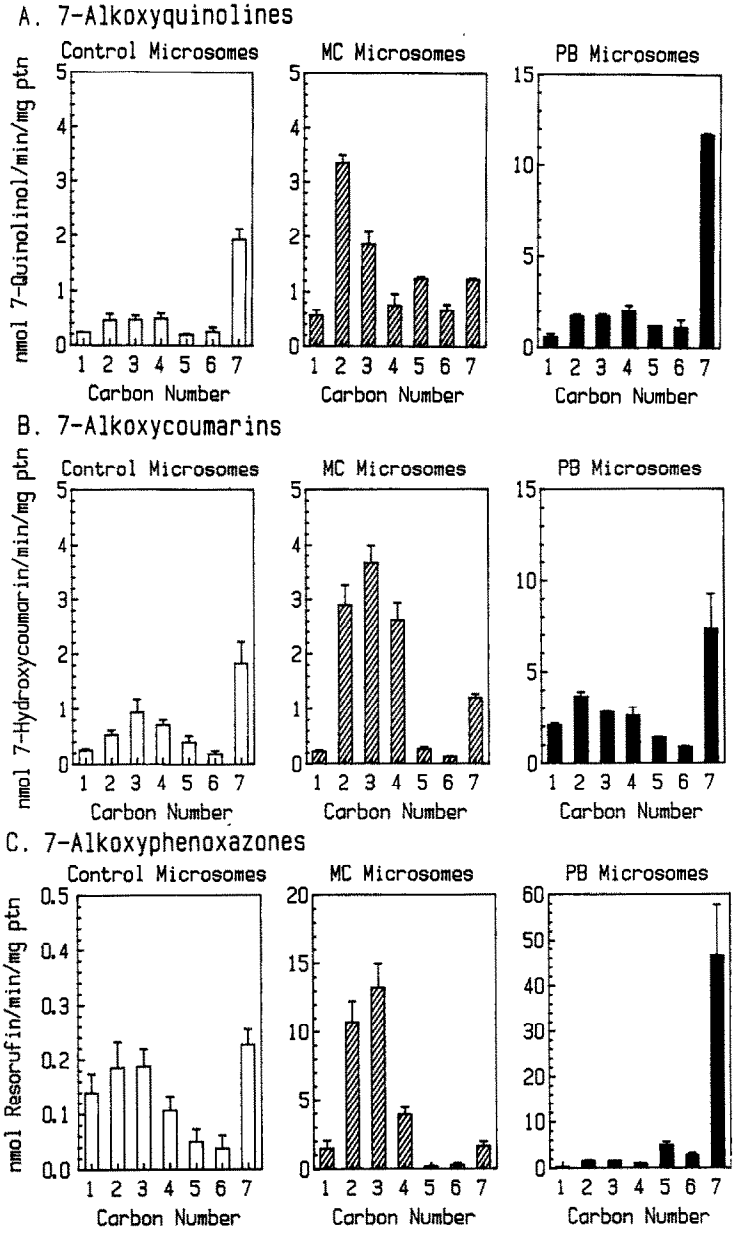

Fig. 8. Structure–activity relationships for 7-alkoxyquinolines, -coumarins, and -phenoxazones. Conditions are given in Materials and Methods. The numbers beneath each histogram bar indicate the length and type of the sidechain. Carbon 7 indicates the 7-benzyloxy substrates. Four to eight rat livers for each treatment group were measured individually. Values are means ± SD.

Table 1. Induction of microsomal O-dealkylation of 7-alkoxyquinolines, -coumarins, and -phenoxazones by PB or MC

Substrate	Fold induction PB	Fold induction MC	Discrimination factor
7-Methoxyquinoline	2.4	2.4	1.0
7-Ethoxyquinoline	3.8	7.5	2.0
7-Propoxyquinoline	3.8	4.0	1.1
7-Butoxyquinoline	4.2	1.6	2.6
7-Pentoxyquinoline	6.3	6.6	1.0
7-Hexoxyquinoline	4.6	2.7	1.7
7-Benzyloxyquinoline	6.1	0.6	10.0
7-Methoxycoumarin	2.1	0.2	10.0
7-Ethoxycoumarin	3.6	2.9	1.2
7-Propoxycoumarin	2.8	3.7	1.3
7-Butoxycoumarin	2.6	2.6	1.0
7-Pentoxycoumarin	1.4	0.2	7.0
7-Hexoxycoumarin	0.9	0.1	9.0
7-Benzyloxycoumarin	7.3	1.2	6.1
7-Methoxyphenoxazone	1.4	10.0	7.5
7-Ethoxyphenoxazone	8.1	58.0	7.1
7-Propoxyphenoxazone	8.0	70.0	8.8
7-Butoxyphenoxazone	8.9	41.0	4.6
7-Pentoxyphenoxazone	101.0	3.3	30.0
7-Hexoxyphenoxazone	74.9	7.2	10.0
7-Benzyloxyphenoxazone	205.0	3.2	64.0

Values were calculated from those obtained in Fig. 8 A, B and C. The discrimination factor compares PB and MC induction for each substrate; it is the degree of induction by an agent giving the greater induction divided by the degree of induction by the agent giving the lesser induction [9]. A discrimination factor of 1 indicates that there is no difference between the two treatment groups in the rate of metabolism for a particular substrate.

ethoxy derivatives, remained slightly elevated for the propoxy and butoxy, then dropped for the pentoxy and hexoxy substrates (Fig. 8A). Activity was highest (3- to 4-fold that of the other quinolines) for 7-benzyloxyquinoline with control microsomes. 7-Alkoxyquinoline O-dealkylation patterns for PB microsomes were similar to those observed for controls except that the rates were 2- to 6-fold higher. The metabolic pattern for MC microsomal O-dealkylation with the quinolines was quite different. Activity was low for the 7-methoxyquinoline and then increased 4- to 5-fold to its highest level with 7-ethoxyquinoline; thereafter, the activities declined. Fold induction values are presented in Table 1, where it is obvious that the greatest induction over control microsomes is recorded for 7-ethoxyquinoline with MC microsomes. However, there was only a discrimination factor of 2 between PB and MC microsomal activities, suggesting that it would be difficult to distinguish PB and MC forms of cytochrome P450. 7-Benzyloxyquinoline with a discrimination factor of 10 was the best quinoline substrate to distinguish between PB and MC cytochrome P450 forms.

The results we obtained for the SARs with 7-alkoxycoumarins (Fig. 8B) were similar to those reported by Matsubara *et al.* [16, 18] for the 7-methoxy- through 7-butoxycoumarins. Metabolic activity for control microsomes increased steadily from the 7-methoxy- to 7-propoxycoumarin and then decreased in an almost linear fashion to the 7-hexoxycoumarin (Fig. 8B). 7-Benzyloxycoumarin had the highest activity of the 7-alkoxycoumarin substrates with control microsomes. O-Dealkylation patterns for the 7-alkoxycoumarins with MC microsomes were similar to those observed for controls, i.e. there was an increase in O-dealkylation rates up to 7-propoxycoumarin and then a decline to the 7-hexoxycoumarin. MC microsomal O-dealkylation rates for the 7-methoxy-, 7-pentoxy- and 7-hexoxycoumarins were lower than those recorded for control microsomes (see induction data in Table 1). 7-Benzyloxycoumarin O-dealkylase activity was higher than that of the 7-methoxy-, 7-pentoxy-, and 7-hexoxycoumarins but lower than that of the 7-ethoxy-, 7-propoxy-, and 7-butoxycoumarins. PB microsomal O-dealkylation rates increased in going from the 7-methoxycoumarin to the 7-ethoxycoumarin and then decreased to the lowest activity observed with 7-hexoxycoumarin. In the coumarin series of substrates the greatest activity and induction was observed for 7-benzyloxycoumarin with PB microsomes. Although 7-methoxy-, 7-pentoxy-, and 7-hexoxycoumarins had larger discrimination factors (Table 1) than the 7-benzyloxycoumarin, they may have less utility in microsomal suspensions or cell homogenates as the MC metabolic rates for the former substrates were less than those observed for constitutive microsomal activities.

There have been several reports on the metabolism of 7-alkoxyphenoxazones by various cytochrome P450 monooxygenases [10, 11, 18, 23, 28–30]. As expected, metabolic activities varied among the reports due to the different assay conditions, enzyme sources, and enzyme preparations. O-Dealkylation of the 7-alkoxyphenoxazones in control microsomal suspensions increased slightly from the 7-methoxy- to the 7-ethoxy- and 7-propoxyphenoxazones and then decreased through to the 7-hexoxy derivative (Fig. 8C). Of the 7-alkoxyphenoxazones tested, the benzyloxy derivative was metabolized at the fastest rate for control microsomes. Metabolic activity increased for all of the phenoxazone derivatives with MC microsomes. 7-Propoxyresorufin had the fastest O-dealkylation rate, with 7-ethoxyresorufin a close second. Induction of O-dealkylase activity followed the same order as the metabolic activities for the *n*-alkoxyphenoxazones with the 7-propoxy and 7-ethoxy ethers being induced 70- and 58-fold, respectively, over control values (Table 1). The metabolic pattern for PB microsomes was quite different from that seen for MC O-dealkylation. Whereas the pentoxy, hexoxy, and benzyloxy derivatives produced the lowest activities with MC microsomal suspensions, these substrates were metabolized the fastest with PB microsomes (Fig. 8C). Again, induction levels followed metabolic activities with the pentoxy, hexoxy, and benzyloxy ethers yielding 101-, 74-, and 205-fold induction levels respectively (Table 1). These latter phenoxazone ethers also had the largest discrimination factors at 30, 10, and 64.

Kinetic studies. Apparent kinetic data for the various fluorescent substrates are presented in Table 2. Linear Lineweaver–Burk plots were obtained for all of the substrates for the concentration ranges tested.

Table 2. Kinetic data for control, MC, and PB microsomes with 7-alkoxyquinolines, -coumarins, and -phenoxazones

	Control			MC			PB		
	K_m (μM)	V_{max}*	K_{cat}† (min^{-1})	K_m (μM)	V_{max}*	K_{cat}† (min^{-1})	K_m (μM)	V_{max}*	K_{cat}† (min^{-1})
7-Methoxyquinoline	71 ± 5	0.37 ± 0.02	0.39 ± 0.02	14.0 ± 0.50	0.35 ± 0.01	0.28 ± 0.01	22.0 ± 5.0	0.91 ± 0.13	0.42 ± 0.06
7-Ethoxyquinoline	773 ± 145	2.1 ± 0.1	3.4 ± 0.24	5.4 ± 0.68	2.14 ± 0.19	2.30 ± 0.21	46.0 ± 4.0	4.4 ± 0.68	2.6 ± 0.40
7-Propoxyquinoline	151 ± 27	1.0 ± 0.1	1.7 ± 0.13	2.1 ± 1.20	1.5 ± 0.27	1.59 ± 0.29	21.0 ± 8.8	2.5 ± 0.72	2.7 ± 0.77
7-Butoxyquinoline	80 ± 8	0.95 ± 0.11	1.6 ± 0.18	0.47 ± 0.06	0.83 ± 0.09	0.89 ± 0.09	13.0 ± 4.2	12.4 ± 1.70	7.3 + 1.0
7-Pentoxyquinoline	84 ± 47	0.7 ± 0.39	1.1 ± 0.64	1.2 ± 0.74	0.53 ± 0.02	0.57 ± 0.02	29.0 ± 0.6	2.0 ± 0.29	1.2 ± 0.17
7-Hexoxyquinoline	202 ± 42	0.46 ± 0.15	0.69 ± 0.31	1.5 ± 0.61	0.29 ± 0.24	0.29 ± 0.27	3.1 ± 2.7	1.0 ± 0.39	0.57 ± 0.24
7-Benzyloxyquinoline	111 ± 67	8.4 ± 3.6	9.2 ± 5.2	2.1 ± 0.52	2.7 ± 1.22	2.0 ± 1.5	2.8 ± 0.9	3.6 ± 0.77	2.9 ± 1.2
7-Methoxycoumarin	46 ± 20	0.22 ± 0.06	0.25 ± 0.06	3.7 ± 0.53	0.25 ± 0.07	0.27 ± 0.05	15.0 ± 6.4	0.68 ± 0.11	0.31 ± 0.05
7-Ethoxycoumarin	37 ± 9	0.39 ± 0.05	0.42 ± 0.06	1.9 ± 0.56	1.4 ± 0.21	1.2 ± 0.05	6.9 ± 2.6	0.90 ± 0.31	0.40 ± 0.13
7-Propoxycoumarin	206 ± 69	1.3 ± 0.29	1.5 ± 0.41	0.89 ± 0.23	2.9 ± 0.37	2.4 ± 0.04	6.9 ± 2.7	0.69 ± 0.18	0.31 ± 0.08
7-Butoxycoumarin	54 ± 11	0.77 ± 0.34	0.94 ± 0.41	1.1 ± 0.63	1.9 ± 0.11	1.7 ± 0.13	5.1 ± 3.0	0.68 ± 0.11	0.30 ± 0.06
7-Pentoxycoumarin	264 ± 121	3.9 ± 2.2	4.6 ± 2.80	3.4 ± 2.52	0.10 ± 0.07	0.04 ± 0.02	3.7 ± 0.7	0.51 ± 0.24	0.23 ± 0.11
7-Hexoxycoumarin	170 ± 68	0.57 ± 0.24	0.65 ± 0.25	48.0 ± 29.0	0.23 ± 0.01	0.19 ± 0.01	11.5 ± 5.7	0.55 ± 0.35	0.25 ± 0.15
7-Benzyloxycoumarin	69 ± 27	4.4 ± 2.3	4.8 ± 2.25	1.6 ± 0.34	1.0 ± 0.14	0.99 ± 0.30	1.2 ± 0.2	5.5 ± 0.52	2.6 ± 0.18
7-Methoxyphenoxazone	0.18 ± 0.06	196.0 ± 65.0‡	0.21 ± 0.06	0.59 ± 0.57	4.42 ± 1.58	3.9 ± 1.56	0.76 ± 0.86	0.48 ± 0.45	0.21 ± 0.19
7-Ethoxyphenoxazone	0.06 ± 0.04	86.0 ± 47.0‡	0.10 ± 0.06	0.05 ± 0.03	14.1 ± 10.0	12.0 ± 8.53	0.20 ± 0.19	0.52 ± 0.36	0.23 ± 0.16
7-Propoxyphenoxazone	0.10 ± 0.04	126.0 ± 19.0‡	0.14 ± 0.02	0.09 ± 0.03	22.0 ± 1.1	19.0 ± 2.49	0.17 ± 0.04	0.82 ± 0.17	0.36 ± 0.07
7-Butoxyphenoxazone	0.07 ± 0.02	51.0 ± 12.0‡	0.06 ± 0.01	0.11 ± 0.07	6.2 ± 0.94	5.5 ± 1.07	0.18 ± 0.06	0.70 ± 0.14	0.31 ± 0.07
7-Pentoxyphenoxazone	0.38 ± 0.25	65.0 ± 30.0‡	0.07 ± 0.03	0.56 ± 0.00	0.38 ± 0	0.28 ± 0	0.22 ± 0.11	3.9 ± 1.4	1.7 ± 0.62
7-Hexoxyphenoxazone	0.12 ± 0.02	4.2 ± 0.5‡	<0.01	0.13 ± 0.10	0.01 ± 0	0.01 ± 0	0.34 ± 0.15	0.57 ± 0.19	0.25 ± 0.08
7-Benzyloxyphenoxazone	0.17 ± 0.10	210.0 ± 44.0‡	0.23 ± 0.04	0.05 ± 0.01	1.6 ± 0.23	1.4 ± 0.07	0.13 ± 0.01	21.0 ± 7.01	9.5 ± 3.3

Values are the means ± SD of four to eight determinations on individual liver preparations for each treatment group. Conditions were as described in Materials and Methods or in the legend of Fig. 8.

*V_{max} values are in nmol product/min/mg protein unless otherwise stated.

†Determined from V_{max} values; K_{cat} = turnover number.

‡V_{max} values are in pmol/min/mg protein.

Apparent K_m values for 7-alkoxyquinolines ranged from 71 to 773 μM for control microsomes. Apparent K_m values for the quinoline ethers obtained with MC and PB microsomes were reduced considerably and ranged from 0.47 to 14 μM for the MC groups and 2.8 to 46 μM for the PB groups. Apparent V_{max} and K_{cat} (turnover number) values generally paralleled the trends observed at saturating substrate levels used for the SAR studies. Exceptions to this were the V_{max} and K_{cat} values for 7-benzyloxyquinoline where higher values were calculated for the control and MC microsomes and lower values obtained for PB microsomes.

The 7-alkoxycoumarins yielded apparent K_m values of 37 (7-ethoxycoumarin) to 265 μM (7-pentoxycoumarin) for the control microsomes (Table 2). The highest V_{max} values for control microsomes were obtained for the 7-propoxy-, 7-pentoxy-, and 7-benzyloxycoumarins (V_{max} = 1.3, 3.9, and 4.4 nmol 7-hydroxycoumarin/min/mg protein respectively); the 7-propoxy- and 7-benzyloxycoumarins correlated with results obtained for the SAR measurements, whereas the 7-pentoxycoumarin did not (Fig. 8B). The K_{cat} values were similar to those for V_{max}. Apparent K_m values for the MC and PB microsomes were significantly smaller than those of the controls. The apparent K_m values for the MC microsomes ranged from 0.89 μM for the 7-propoxycoumarin to 48 μM for the 7-hexoxycoumarin. Apparent K_m values for the PB microsomes were from 1.2 μM for the 7-benzyloxy- to 15μM for the 7-methoxycoumarin. The apparent V_{max} and K_{cat} values (Table 2) for MC and PB microsomes followed the same trends seen in the SAR studies (Fig. 8B), i.e. 7-ethoxy-, 7-propoxy-, and 7-butoxycoumarins gave the largest V_{max} values (V_{max} = 1.4, 2.9, and 1.9 nmol 7-hydroxycoumarin/min/mg protein respectively) for MC microsomes, while 7-ethoxy- and 7-benzyloxycoumarins gave the largest V_{max} values (V_{max} = 0.90 and 5.5 nmol 7-hydroxycoumarin/min/mg protein respectively) with the PB microsomes.

The apparent K_m values for the 7-alkoxyphenoxazones were all within the same range, i.e. <1 μM (Table 2). This was expected; Burke and Mayer [10] reported that there were only small differences in apparent K_m values for 1C to 6C in the sidechains of phenoxazone derivatives with murine control, PB, and MC hepatic microsomes. The V_{max} and K_{cat} values for all of the treatment groups followed the same trends established in the SAR studies (Fig. 8C). 7-Methoxy-, 7-ethoxy-, 7-propoxy-, and 7-benzyloxyphenoxazones had the highest V_{max} values in the control microsomes (V_{max} = 196, 86, 126 and 210 pmol resorufin/min/mg protein respectively). With MC microsomes, 7-ethoxy- and 7-propoxyphenoxazones yielded the highest V_{max} values (V_{max} = 14 and 22 nmol resorufin/min/mg protein respectively); these values are 164- and 176-fold the values obtained for these substrates with control microsomes. 7-Pentoxy- and 7-benzyloxyphenoxazones gave the highest V_{max} values with PB microsomes (V_{max} = 3.9 and 21 nmol resorufin/min/mg protein respectively); these values were 59- and 101-fold those obtained with control microsomal suspensions.

DISCUSSION

There is a need for better methods to assay for specific cytochrome P450 variants in microsomal suspensions or in purified preparations [10, 31]. Determination of the metabolic contributions of specific cytochrome P450 variants in microsomal suspensions can be complex, while site-directed mutagenesis and subsequent determination of catalytic activities with only one or two substrates may result in limited information on the catalytic centers. Research on catalytic activities using mutant strains of cytochrome P450 could be more meaningful if a series of substrates were available that retained the same primary structure but with the substitute groups modified slightly. Structural changes in the cytochrome P450 variants could then be correlated more exactly in regard to size and hydrophobicity of the catalytic centers.

Information has been presented on the synthesis, binding and metabolic characteristics of 7-alkoxyquinolines. These new fluorescent substrates have been compared with other coumarin and phenoxazone substrates that have the same sidechain substituents. Three of the coumarin substrates (7-pentoxy-, 7-hexoxy-, and 7-benzyloxycoumarin) have not been reported on previously. The quinolines were prepared for the purpose of determining if the N in the ring system conferred special properties on the molecule that resulted in any metabolic specificity.

The quinolines are interesting in that the 7-quinolinol exists in a prototropic equilibrium in aqueous solutions. Mason *et al.* [26] have shown that four ionic species exist in aqueous solution and the phenolic group is more acidic and the ring nitrogen more basic in the excited state than in the ground state of the molecule. A two-stage prototropic change in the excited state from the neutral molecule to the zwitterion was proposed in neutral solutions, while a one-stage prototropic change was proposed for acid and alkaline solutions [26]. Fluorescence, then, does not occur only from a single species of molecule in the excited state as it does for resorufin and 7-hydroxycoumarin. The pH titration curves in Fig. 2 reflect the transitions between the different species and suggest that a more sensitive assay technique may be developed that takes advantage of the higher fluorescence of 7-quinolinol in acid and alkaline environments.

The spectral interactions observed upon binding of the various quinolines to PB microsomal suspensions (Figs. 4 and 5) deserve some comment. There is a transition in the spectral interactions in going through the series of quinolines, i.e. the 7-methoxy- to the 7-hexoxyquinoline; the type of spectral interaction observed changes from a Reverse Type I spectrum for the 7-methoxyquinoline to a Type I spectrum for the 7-hexoxyquinoline at concentrations of 80 μM (Figs. 6 and 7). Intermediate spectra (a combination of Type I and Reverse Type I) were observed for the 2C to the 4C compounds. Only Reverse Type I spectra were recorded at concentrations of 8 μM or less for any of the quinoline substrates with PB microsomes. Type I and Reverse Type I spectra are thought to be the result of a conversion of high spin cytochrome P450 com-

ponents to low spin states and vice versa [27]. Microsomal suspensions probably contain a mixture of high and low spin components. It is conceivable that 7-methoxyquinoline would bind only to the high spin components and convert a large portion of these components to low spin states. Extension of the sidechain could increase the amount of binding to low spin components with subsequent conversion to high spin states. Differences in the binding constants of the various quinolines to high and low spin cytochrome P450 components would result in intermediate spectra. A high affinity to high spin components would also account for the observation that only the Reverse Type I spectrum is observed below 8 μM.

The results with the SAR studies (Fig. 8) indicate that the 7-alkoxyquinolines are widely metabolized and that the metabolism can be induced by chemicals (Table 1). Comparison of the SARs and induction levels for the quinolines with those of coumarins and phenoxazones indicates that the quinolines are better substrates in general for PB and MC microsomes than the coumarins but worse than the phenoxazones. Metabolic activities for the coumarins and quinolines are within the same ranges for all of the microsomal groups. Only with the phenoxazones does one see dramatic differences (i.e. >10-fold) in metabolic activities and induction levels. Large differences were observed in some of the kinetic data for the substrates (Table 2). Again, some of the most striking differences exist between the resorufins and the other two substrates. Control K_{cat} and V_{max} values for the phenoxazones were generally lower than those for the coumarins and quinolines. In the MC treatment groups the 7-ethoxy- and 7-propoxy-, and 7-butoxyphenoxazone K_{cat} and V_{max} values were much higher than those of the comparable coumarin and quinoline analogs. In the PB treatment groups, the 7-pentoxy- and 7-benzyloxyphenoxazones and the 7-ethoxy-, 7-propoxy-, and 7-butoxyquinolines had high K_{cat} and V_{max} values as compared with similar substrates studied here.

Whether or not the N in the quinoline ring system is the cause of these differences is unknown at this time. Other investigations are currently underway to determine the reactivity of these substrates with several purified cytochrome P450 variants. Preliminary investigations with selective inducers of coumarin hydroxylases [32] such as pyrazine, pyrazole, and aminotriazole with DBA/2J Han mice indicate that there is a specific induction of 7-ethoxyquinoline O-dealkylase activity (unpublished data). Perhaps the contribution of the ring N in quinoline will be more evident from these studies. In the meantime, the 7-alkoxyquinolines appear to be a useful series of substrates for the assay of cytochrome P450 monooxygenases.

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