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# Pyridine Effects on Expression and Molecular Regulation of the Cytochrome P450IA Gene Subfamily

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## SUMMARY

The expression and molecular regulation of the cytochrome P450IA (P450IA) gene subfamily have been examined in rat hepatic tissue after treatment with pyridine. The microsomal ethoxyresorufin *O*-deethylase activity, which has been shown to be specific for the P450IA subfamily, was increased ~2- and 3.5-fold over control values at 10 and 16 hr, respectively, after a single dose of pyridine (100 mg/kg, intraperitoneally). P450IA1 protein expression was also elevated in a time-dependent manner, with a maximal increase in P450IA1 protein being seen at ~16 hr after a single dose of pyridine (100 mg/kg, intraperitoneally), as detected by immunoblot analysis using a monoclonal antibody that detects both P450IA1 and P450IA2. The immunochemically detectable level of P450IA1 decreased to that of control at 48 hr after treatment. Oligonucleotide probes specific for P450IA1 and P450IA2 mRNA were used in hybridization analyses to examine mRNA levels of P450IA1 and P450IA2, respectively. The level of P450IA1 mRNA in poly(A)$^+$ mRNA was increased ~3- and 2-fold at 5 and 12 hr, respectively, after a single injection of pyridine, as evidenced by both slot blot and Northern blot analyses. A lesser increase (~1.5–2-fold) in P450IA2 mRNA was also seen at 5 and 12 hr after treatment. The P450IA1 and P450IA2 mRNA levels returned to control values at 48 hr after pyridine administration. These results were compared with those produced by 3-methylcholanthrene at 5 hr after treatment. A multiplex polymerase chain reaction assay was also used to monitor simultaneously the changes in P450IA1, P450IA2, and P450IIE1 mRNA levels, and the results showed induction of P450IA1, in agreement with the results of slot and Northern blot analyses. In summary, metabolic activity assays, immunochemical detection, and Northern and slot blot analyses provide evidence to support the conclusion that pyridine modulates the expression of the P450IA gene subfamily and does so by elevating P450IA1 and P450IA2 mRNAs, through either transcriptional activation or increased mRNA stabilization. These results are in sharp contrast to P450IIE1 induction by pyridine, which appears to proceed through increased translational efficiency. Thus, pyridine, which is present in tobacco and tobacco smoke, is capable of simultaneously elevating multiple forms of P450 that are active in carcinogen metabolism.

Chronic pretreatment of rats and rabbits with pyridine has been shown to result in increased hepatic P450 content. The induction of P450IIE1 by pyridine was demonstrated by metabolic activity and immunoblot analysis (1–3). In recent studies, we reported that P450IIE1 was rapidly increased after the administration of a single dose of pyridine (3, 4). The rapid induction of P450IIE1 protein by pyridine was attributed to enhanced protein synthesis, as shown by [$^{14}$C]leucine incorporation (3), and increased translational efficiency, as evidenced by a lack of transcriptional activation, a small decrease in P450IIE1 poly(A)$^+$ mRNA, and a redistribution of P450IIE1 mRNA to larger polyribosomes (4).

In the rat liver, P450IA1 and P450IA2,[1] in the P450IA gene subfamily, are the major enzymes induced in response to PAH such as 3-MC, β-naphthoflavone, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (5). P450IA1 is also known to be induced by cigarette smoke (6). Both genes are transcriptionally activated in response to PAH exposure (7). P450IA1 exhibits significant activity in the metabolism of PAH carcinogens; in contrast, P450IA2 is less active in PAH metabolism but has a high degree of activity in the metabolism of aflatoxin (8).

Pyridine is an amphipathic solvent that is widely employed in industry and is also a constituent of tobacco and tobacco smoke (9). Because pyridine is readily absorbed through inhalation, ingestion, or cutaneous exposure, the effects of this agent on expression of the P450IA gene subfamily of rat liver

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[1] P450IA1 (gene *CYPIA1*) and P450IA2 (gene *CYPIA2*) is the nomenclature recommended for members of this gene subfamily, which includes P-450c and P-450d in rat and P-450LM$_6$ and P-450LM$_4$ in rabbit, respectively (25).

**ABBREVIATIONS:** P450, cytochrome P450; EROD, ethoxyresorufin *O*-deethylase; PAH, polycyclic aromatic hydrocarbon(s); 3-MC, 3-methylcholanthrene; RT, reverse transcriptase; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

P450s were examined as part of a comprehensive evaluation of P450 induction with emphasis on those P450s associated with carcinogen metabolism.

In this study, expression of P450IA1 and P450IA2 was monitored after pyridine treatment, using EROD activity and Western blot analysis. Examination of P450IA1 and P450IA2 mRNA levels in poly(A)$^+$ mRNA isolated from rat hepatic tissue after pyridine treatment, using slot and Northern blot analyses and a multiplex PCR assay, revealed a notable time-dependent increase in P450IA1 mRNA and a slight increase in P450IA2 mRNA levels. Thus, the P450IA gene subfamily of rat liver P450 is rapidly induced by pyridine, as demonstrated by enhanced metabolic activity and increased expression of P450IA1 and P450IA2 protein and mRNA. These results suggest that pyridine modulates the expression of different gene subfamilies (P450IA, P450IIE) of rat hepatic P450 by separate mechanisms and induces several forms of P450 that are active in carcinogen metabolism.

## Materials and Methods

**Chemicals.** Pyridine was purchased from Fisher Scientific Company (Fair Lawn, NJ). Nitrocellulose was purchased from Bio-Rad (Richmond, CA). Biotinylated donkey anti-goat IgG and streptavidin-conjugated horseradish peroxidase were purchased from BRL (Gaithersburg, MD). Avian myeloblastosis virus RT was obtained from United States Biochemicals (Cleveland, OH). Random primer and RNasin were purchased from Pharmacia-LKB (Piscataway, NJ) and NEB (Beverly, MA), respectively.

**Animals and treatment.** Male Sprague-Dawley rats (110–150 g) were treated with pyridine (100 mg/kg, intraperitoneally) and sacrificed at various times after treatment or treated with 3-MC and sacrificed at 5 hr (for mRNA) or at 24 hr (for microsomes). Animals were fasted for 16 hr before sacrifice for either mRNA isolation or preparation of microsomes. Livers used for the preparation of microsomes were perfused with isotonic saline, as described previously (10). Each data point consisted of samples pooled from two or three animals, and a total of four groups of animals were used in the experiments.

**Isolation of microsomal proteins.** Hepatic microsomes, isolated by differential centrifugation, were washed in pyrophosphate buffer and stored in 50 mM Tris-acetate buffer (pH 7.4), containing 1 mM EDTA and 20% glycerol, at −70° until used (10). Protein was assayed by the method of Lowry *et al.* (11).

**EROD activity.** Microsomal EROD activity was measured at 37° in a Perkin Elmer LS-5 fluorescence spectrophotometer (12). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.8), 0.5 μM ethoxyresorufin, 0.4 mg of microsomal suspension, and 0.25 mM NADPH, in a total volume of 2.0 ml. A baseline of fluorescence was recorded at an excitation wavelength of 510 nm and an emission wavelength of 586 nm. Reactions were initiated by NADPH addition after a 2-min preincubation period, and the progressive increase in fluorescence associated with the deethylation of ethoxyresorufin was recorded.

**Monoclonal antibodies for P450IA1 and P450IA2.** The monoclonal antibody that recognized both P450IA1 and P450IA2 and that was used in these studies was kindly provided by Drs. H. V. Gelboin and S. S. Park from the National Institutes of Health/National Cancer Institute (Bethesda, MD).

**Immunoblot analysis.** Microsomal proteins were separated by 7.5% SDS-PAGE and electrophoretically transferred to nitrocellulose paper. After a 1-hr incubation in blocking solution (phosphate-buffered saline with 5% nonfat Carnation powdered milk) at 37°, the nitrocellulose membrane was incubated with monoclonal antibody MC 1-7-1, which detects both P450IA1 and P450IA2. Biotinylated donkey anti-mouse IgG was used as the secondary antibody, and this was followed by incubation with streptavidin-horseradish peroxidase, as described previously (3, 4). Immunoreactive protein was visualized with 4-chloro-1-naphthol.

**Isolation of total RNA and poly(A)$^+$ mRNA.** Total RNA was isolated using direct lithium chloride precipitation, according to the method of Cathala *et al.* (13). One gram of hepatic tissue was homogenized in 10 ml of lysis buffer (5 M guanidine thiocyanate, 5 mM EDTA, 50 mM HEPES, pH 7.5, 0.1 M β-mercaptoethanol), using a Polytron homogenizer. After centrifugation at 9000 × *g* to remove cell debris, the solution was mixed with 4–5 volumes of 4 M LiCl and left to stand overnight at 4°. The RNA-enriched pellet was obtained by centrifugation at 9000 × *g* at 4° for 30 min and was resuspended in 5 ml of lysis buffer. RNA was then extracted with phenol/chloroform (1:1) twice, followed by extraction with chloroform. The RNA in the aqueous phase was precipitated with ethanol and resuspended in diethylpyrocarbonate-treated water. Poly(A)$^+$ mRNA was isolated from total RNA using an oligo(dT)-cellulose column, according to the method of Jacobson (14).

**P450IA1 and P450IA2 oligonucleotide probe preparation.** The oligonucleotide probes, which have been shown to be specific for P450IA1 and P450IA2 by Giachelli and Omiecinski (15), were obtained from Research Genetics (Huntsville, AL). The sequences are 5′-d(TCTGGTGAGCATCCAGGACA)-3′ and 5′-d(GGAAAAGGAACAAGGGTGGC)-3′ for P450IA1 and P450IA2, respectively. These oligonucleotides are complementary to region 1650–1669 in the P450IA1 cDNA sequence and to region 1563–1582 in the P450IA2 cDNA sequence, respectively.

**RNA slot blot hybridization.** RNA slot blot analysis was performed using a Schleicher & Schuell slot blot apparatus (Minifold II). Total RNA or poly(A)$^+$ mRNA was serially diluted in 15× standard saline citrate (1× standard saline citrate is 150 mM NaCl, 15 mM sodium citrate, pH 7.4) and applied to slots according to the manufacturer's protocol. The transfer membrane was baked in a vacuum oven at 80° for 2 hr, and the filter was then prehybridized in a solution of 6× SSPE (1× SSPE is 0.15 M NaCl, 10 mM $NaH_2PO_4$, 1 mM $Na_2EDTA$, pH 7.4) containing 0.1% SDS and 5× Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrrolidine, 0.1% bovine serum albumin (pentex fraction V)], without probe, for 2–6 hr at 57°, in a heat-sealable bag. The prehybridization solution was replaced with hybridization solution consisting of 4 ml of prehybridization solution, 1 ml of 50% (w/v) dextran sulfate, 125 μl of 10 mg/ml sonicated salmon sperm DNA, and 2–10 × $10^6$ cpm of $^{32}$P-end labeled probe/filter. The hybridization was performed at 57° for 18–20 hr. Filters were washed at the hybridization temperature for 1 hr, with three changes of a solution of 6× SSPE and 0.1% SDS, followed by autoradiography with Kodak X-Omat AR film at −80° in a cassette containing intensifying screens.

**Northern blot hybridization.** Northern blotting was carried out according to the procedures described in Schleicher & Schuell molecular biology protocols. Briefly, total and poly(A)$^+$ mRNA isolated from rat livers were resolved by electrophoresis in 1% agarose gels containing 2.2 M formaldehyde and were then transferred to nitrocellulose paper (or GeneScreen Plus; NEN) by capillary transfer. Each membrane was baked under vacuum at 80°, prehybridized, and then incubated in a hybridization solution containing $^{32}$P-end labeled oligonucleotide probes, as described above for slot blot analysis. The results of slot and Northern blot analyses were confirmed in two separate series of animal experiments. Blots were also probed with an oligonucleotide to P450IIE1, which is not induced by transcriptional activation, to ensure that equal amounts of total RNA and poly(A)$^+$ mRNA were loaded onto the agarose gel and transferred to the nitrocellulose paper.

Films were exposed at −80° for 3 hr to 5 days (Northern blots), using Dupont intensifying screens. Slot and Northern blot analyses were performed on different mRNA samples from a total of four different experiments utilizing groups of animals.

**Scanning densitometry.** Scanning densitometry was performed with a Molecular Dynamics computing densitometer. The area of each slot was integrated using ImageQuant Software (version 3.0), followed by background subtraction. The quantitation of the mRNA loaded on the slot was accomplished by hybridization of stripped membranes with

$^{32}$P-end labeled poly(dT)$_{16}$, and the relative change in P450IA1 or P450IA2 mRNA levels was determined from normalization of the P450IA1 or P450IA2 hybridization signal to the mRNA loaded in the slot.

**Multiplex PCR assay**. Total RNA and poly(A)$^+$ mRNA were isolated from the livers of untreated and pyridine-treated (100 mg/kg, intraperitoneally) male Sprague-Dawley rats, at 1, 5, and 12 hr after treatment, as described above. First-strand cDNAs were synthesized using poly(A)$^+$ mRNA (3.0 μg), Avian myeloblastosis virus RT (0.5 unit/μl), random primer (50 ng/μl), 2.0 mM of each of four nucleotide triphosphates, RNasin (0.8 unit/μl), and RT buffer (50 mM Tris·HCl, pH 8.3, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithiothreitol), in a total reaction volume of 60 μl. One sixtieth of the reaction product served as template for each PCR reaction. PCR reactions consisted of 0.1 μM of each of the five primers (Table 1), 0.2 mM of each nucleotide triphosphate, 2 μl of 32.5 mM $MgCl_2$ supplement, 0.5 unit of Amplitaq (*Thermus aquaticus*) polymerase, and 2 μl of 10× PCR buffer (supplied by Perkin-Elmer Cetus), in a total reaction volume of 20 μl. Gene-specific primers were carefully selected from 3′ and 5′ regions of the published cDNA sequences (16–18), to yield individual products that differed in size. Table 1 contains a list of primers used and the size of the amplified products.

PCR reactions were performed in a Perkin Elmer Cetus thermocycler for 20, 30, or 40 cycles, using the following parameters: denaturing at 94° for 1 min, annealing at 50° for 15 sec, and elongation at 72° for 5 min; 30 cycles were found to be optimal. Amplified DNA products were electrophoresed in a 1% agarose gel using a φX174/*Hinc*II ladder as a size marker. The identity of each of the *CYP1A1, CYP1A2*, and *CYP2E1* fragments was confirmed by restriction analysis and by sequencing (results not shown).

## Results

**EROD activity**. EROD activity is primarily associated with forms of P450 (P450IA1 and P450IA2) induced by 3-MC and other PAH and, of these, P450IA1 exhibits the greater activity (~10-fold) in the metabolism of ethoxyresorufin (19). The EROD activity of the hepatic microsomal suspensions isolated from rats treated with a single dose of pyridine (100 mg/kg, intraperitoneally) was examined, and the results were compared with those from untreated animals. EROD activity was elevated ~2- and 3.5-fold over control values at 10 and 16 hr, respectively, after a single dose of pyridine (Fig. 1). The catalytic activity appeared to be maximized at ~16 hr, followed by a decrease to ~2-fold over control at 24 hr after treatment (Fig. 1). In view of this increase in EROD activity, complementary studies were initiated to examine expression of P450IA1 protein, using immunochemical detection.

**Induction of P450IA1 and P450IA2 by pyridine**. A monoclonal antibody that detected both P450IA1 and P450IA2 was used to examine the time-dependent increase in these protein levels in rat hepatic microsomes after a single dose of pyridine. Western blot analysis revealed that pyridine elevated P450IA1 protein levels 2-fold between ~10 and 16 hr, with the maximal increase being seen at 16 hr after pyridine treatment (Fig. 2). These data are in good agreement with the results of the EROD assay. The P450IA1 protein level appeared to decrease significantly at 24 hr, relative to that measured at 16 hr, with a subsequent decline to levels present in untreated animals at 48 hr after treatment. The levels of P450IA2 also increased with time (Fig. 2). P450IA2 levels increased during the period of 10–24 hr, with a maximal elevation of P450IA2 protein being seen at 16 hr. P450IA2 declined to control levels at 48 hr (Fig. 2). In general, these data correlate well with the results of metabolic experiments monitoring EROD activity and provide evidence that the induction of P450IA1 and P450IA2 occurs after pyridine exposure. In subsequent research, the molecular regulation of the P450IA gene subfamily after pyridine treatment was examined.

TABLE 1
**PCR primers and products**

| P450 gene | 5′ primer | 3′-primer | Product size |
|---|---|---|---|
| | | | *bp* |
| *CYP1A1* | TGACCTCTTTGGAGCT | TTGAGCCTCAGCAGAT | 1050 |
| *CYP1A2* | TGACCTCTTTGGAGCT | GACGTGTTCACAGGTT | 600 |
| *CYP2E1* | TCTGAGGCTCATGAGT | GACAGGAGCAGAAACA | 770 |

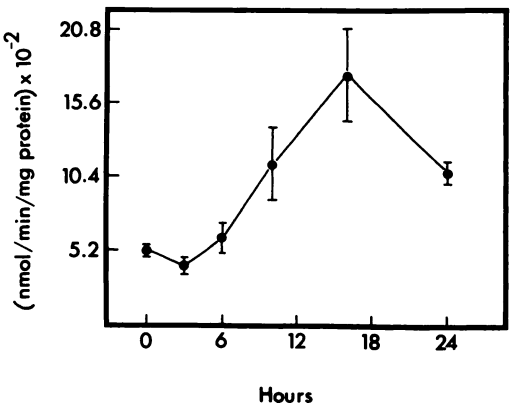


**Fig. 1.** Time-dependent increase in microsomal EROD activity after a single dose of pyridine (100 mg/kg, intraperitoneally).

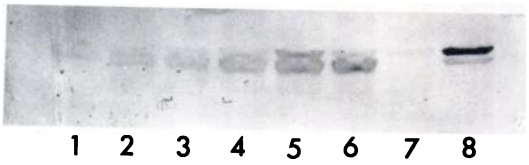


**Fig. 2.** Western blot analysis of the time-dependent increase in P450IA1 and P450IA2 after pyridine treatment. *Lanes 1–7*, 30 μg of microsomal protein from pyridine-treated animals; *lane 8*, 15 μg of microsomal protein from 3-MC-treated animals. The protein bands present in *lanes 1–7* represent the level of immunochemically detectable P450IA1 (*top band*) and P450IA2 (*bottom band*) protein monitored at 0, 3, 6, 10, 16, 24, and 48 hr after a single dose of pyridine, respectively. For comparison, *lane 8* contains microsomal protein isolated from animals at 24 hr after 3-MC treatment (25 mg/kg, intraperitoneally).

**Hybridization analysis of P450IA1**. Slot blot analysis of P450IA1 mRNA levels was performed using poly(A)$^+$ mRNA isolated from hepatic tissue at 0, 1, 5, 12, 24, and 48 hr after a single injection of pyridine, using a 20-mer oligonucleotide probe complementary to P450IA1 mRNA (Figs. 3 and 4). The slot blot analysis revealed that the levels of P450IA1 mRNA in poly(A)$^+$ mRNA decreased slightly at 1 hr and increased ~3- and 2-fold at 5 and 12 hr, respectively, after pyridine treatment, as judged from scanning laser densitometry. Since the slot blots at the 0 and 1 hr time points show nonspecific hybridization, the level of P450IA1 mRNA was also examined using Northern blot analysis of total and poly(A)$^+$ mRNA (Fig. 5) in order to demonstrate specificity for relative changes. The oligonucleotide probe recognized a single P450IA1 mRNA transcript, having a molecular size of 2.7 kilobases, in both total (not shown) and poly(A)$^+$ mRNA preparations. Northern blot analysis confirmed the time-dependent increase of P450IA1 mRNA in both total RNA (not shown) and poly(A)$^+$ mRNA preparations, with an ~3-fold increase being observed at 5 and 12 hr, respectively (Fig. 5). In a second series of experiments, in which the slot

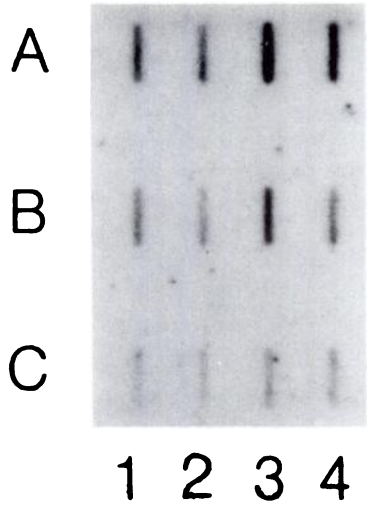


**Fig. 3.** Slot blot hybridization using a 20-mer synthetic P450IA1 oligonucleotide to probe poly(A)$^+$ mRNA isolated from rat livers at 0, 1, 5, and 12 hr, (*lanes 1–4*, respectively), after a single dose of pyridine (100 mg/kg, intraperitoneally). Samples contained in *slots A–C* represent serial dilutions of poly(A)$^+$ mRNA from 1.8 to 0.45 $\mu$g. The time-dependent increase of P450IA1 mRNA was noted in both total and poly(A)$^+$ mRNA, with the maximal increase occurring at ~5 hr (*lane 3*). Approximately 3- and 2-fold increases in P450IA1 poly(A)$^+$ mRNA levels occurred at 5 and 12 hr, respectively, as suggested by scanning laser densitometry.

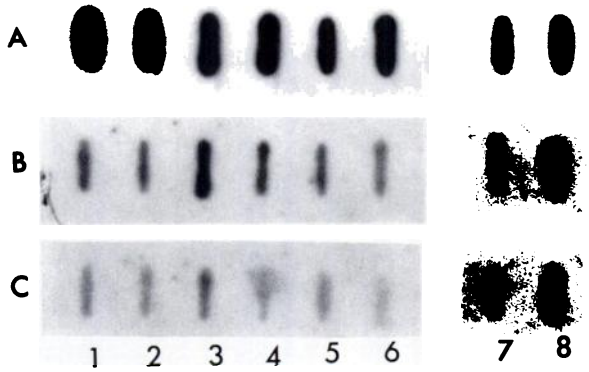


**Fig. 4.** Slot blot analyses of P450IA1 and P450IA2 mRNA levels in rats after a single dose of pyridine (100 mg/kg, intraperitoneally). Poly(A)$^+$ mRNA (1.4 $\mu$g) isolated from rats at 0, 1, 5, 12, 24, and 48 hr after treatment with pyridine was blotted onto *lanes 1–6*, respectively, and probed with an oligonucleotide specific for either P450IA1 or P450IA2. For comparison, *lanes 7* and *8* contain poly(A)$^+$ mRNA from untreated animals and from animals at 5 hr after treatment with 3-MC, respectively. *Row A*, After hybridization with the P450IA1 or P450IA2 oligonucleotide probe, the membrane was stripped and rehybridized with $^{32}$P-labeled poly(dT)$_{16}$, for quantitation of mRNA loaded in the slots. Less than a 10% variation in integrated intensity was monitored using scanning densitometry, with the exception of lane 5, which had 30% less mRNA loaded. *Rows B* and *C*, The membranes were hybridized with $^{32}$P-end labeled oligonucleotide probes for P450IA1 and P450IA2, respectively. These blots showed maximal increases in P450IA1 and P450IA2 mRNA at 5 hr after treatment (3- and 2-fold over control, respectively), which was determined from normalization of individual slot intensities to the respective amount of mRNA added (*row A*).

blot was normalized for mRNA loading using poly(dT)$_{16}$, the same results were obtained (i.e., an increase in P450IA1 mRNA at 5 and 12 hr) (Fig. 4). Moreover, in this series of experiments, the time frame was extended and P450IA1 mRNA was found to decline to the level of untreated animals at 48 hr (Fig. 4). The results obtained with 3-MC at 5 hr after treatment are provided for comparison and show a greater increase in the P450IA1 mRNA level, as compared to that produced by pyridine.

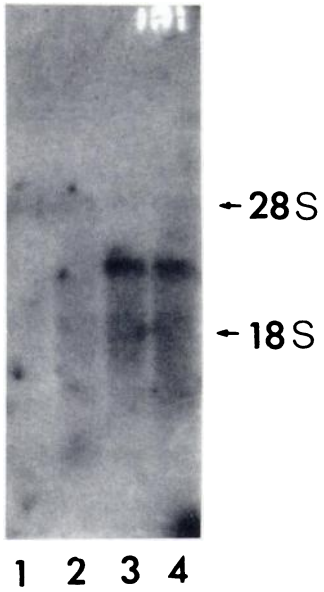


**Fig. 5.** Northern blot analysis using a 20-mer synthetic oligonucleotide for P450IA1 to examine P450IA1 mRNA levels in poly(A)$^+$ mRNA. *Lanes 1–4*, 2 $\mu$g of poly(A)$^+$ mRNA isolated from rat livers at 0, 1, 5, and 12 hr, respectively, after a single dose of pyridine (100 mg/kg, intraperitoneally). The specificity of the probe and the time-dependent increase of P450IA1 mRNA in the poly(A)$^+$ mRNA were noted, with a notable increase observed at the 5-hr time point.

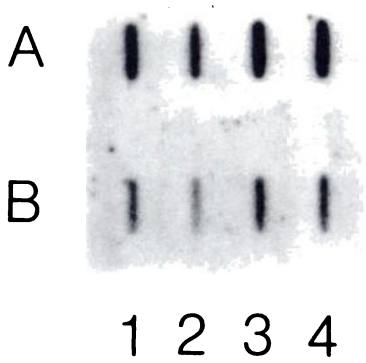


**Fig. 6.** Slot blot hybridization using a 20-mer synthetic P450IA2 oligonucleotide to probe poly(A)$^+$ mRNA isolated from rat livers at 0, 1, 5, and 12 hr (*lanes 1–4*, respectively), after a single dose of pyridine (100 mg/kg, intraperitoneally). *Slots A* and *B*, 1.8 and 0.9 $\mu$g of poly(A)$^+$ mRNA, respectively. A ~2- and 1.5-fold increase, as suggested by scanning laser densitometry, in P450IA2 mRNA levels was noted at 5 and 12 hr, respectively, after treatment.

**Hybridization analysis of P450IA2.** A 20-mer oligonucleotide probe complementary to the P450IA2 mRNA sequence was used to monitor the level of P450IA2 mRNA in poly(A)$^+$ mRNA isolated from rat livers. Fig. 6 shows the slot blot analysis of rat liver poly(A)$^+$ mRNA at 0, 1, 5, and 12 hr after pyridine treatment. A slight decrease in P450IA2 mRNA was seen at 1 hr after treatment, whereas a small (1.5–2-fold) increase in P450IA2 mRNA levels was observed at 5 and 12 hr. The P450IA2 oligonucleotide probe was also utilized in Northern blot hybridization to assess P450IA2 mRNA levels after treatment (Fig. 7). The probe hybridized to the P450IA2 mRNA in the poly(A)$^+$ mRNA with a molecular size of approximately 2.0 kilobases. An ~1.5–2-fold increase occurred at 5 and 12 hr after a single dose of pyridine. P450IA2 levels were found to decline to the levels present in untreated animals at 48 hr (Fig. 4). The elevation of P450IA2 mRNA by pyridine was less than

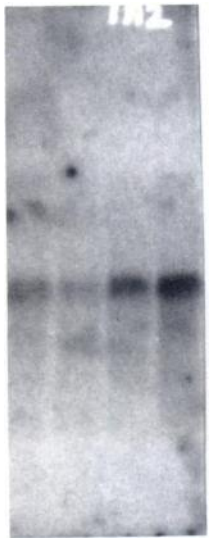


**Fig. 7.** Northern blot analysis using a 20-mer synthetic oligonucleotide for P450IA2 to examine P450IA2 mRNA levels in poly(A)$^+$ mRNA. *Lanes 1–4*, 2 μg of poly(A)$^+$ mRNA isolated from rat livers at 0, 1, 5, and 12 hr, respectively, after a single dose of pyridine (100 mg/kg, intraperitoneally). The specificity of the probe and the time-dependent increase of P450IA2 mRNA in the poly(A)$^+$ mRNA were noted, with the larger increase occurring at the 12-hr time point.

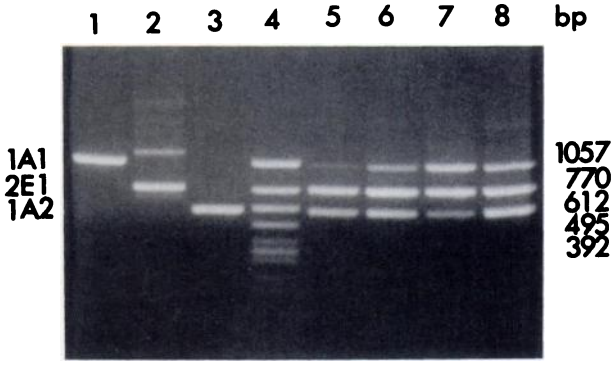


**Fig. 8.** Multiplex PCR analyses of mRNAs for P450IA1, P450IIE1, and P450IA2. *Lanes 1, 2,* and *3*, individual RT PCR products of P450IA1, P450IIE1, and P450IA2, respectively, from 12-hr pyridine-treated animals; the larger fragment (*lane 2*) is an occasional artifact. *Lane 4*, ϕX174/*Hin*cII ladder as a size marker. *Lanes 5–8*, P450IA1, P450IA2, and P450IIE1 RT PCR products obtained from untreated and pyridine-treated animals; *lane 5*, untreated; *lanes 6, 7,* and *8*, 1, 5, and 12 hr after pyridine treatment, respectively. The identity of the fragments as P450IA1, P450IA2, and P450IIE1 was confirmed by restriction analysis and sequencing (not shown).

that produced by a single treatment of 3-MC, as shown by relative comparison of data for 3-MC at 5 hr after treatment (Fig. 4).

**Multiplex PCR assay for P450IA1, P450IA2, and P450IIE1.** As shown in Fig. 8, P450IA1, P450IA2, and P450IIE1 mRNAs may be detected simultaneously using PCR, with the possible exception of the untreated sample, in which the P450IA1 band was extremely faint. This is in agreement with previous observations that P450IA1 mRNA levels are barely detectable in normal hepatic tissue (20). P450IA1 mRNA was clearly elevated by pyridine treatment in a time-dependent manner, with only a slight increase being seen for P450IA2 mRNA. These results are consistent with those obtained from Northern and slot blot analyses. A lesser increase was noted for P450IA2, although an ~2-fold increase in intensity was noted at 12 hr, relative to control. In contrast, P450IIE1 mRNA levels remained comparable at all time points, indicating that pyridine failed to cause transcriptional activation of this gene, consistent with previous observations (3, 4, 21). Thus, this PCR multiplex assay represents a rapid and easy method for detecting and monitoring simultaneously several different gene products in one reaction, in this case P450 mRNAs, and the results are in agreement with those obtained from slot and Northern blot analyses. Inclusion of primers for a gene that is unaffected by transcriptional activation (e.g., *CYP2E1*) permits a relative comparison of mRNA levels in tissues after exposure to a xenobiotic.

## Discussion

Previous work in this laboratory has shown that pyridine elevates hepatic microsomal P450 content in both rats and rabbits (1–3). The induction of P450IIE1 has been examined after either chronic or acute administration of pyridine, and the time- and dose-response associated with pyridine induction of P450IIE1 were characterized (3, 4). Subsequent mechanistic studies suggested increased translational efficiency as being the mechanism initially responsible for the rapid elevation of P450IIE1 (4, 24). Concomitantly, examination of the SDS-PAGE of rabbit or rat hepatic microsomes revealed an apparent increase in the band intensity occurring in the regions of P450IA1 and P450IA2. Thus, studies on the induction of members of the P450IA gene subfamily were conducted for the purpose of examining whether pyridine induces the P450IA subfamily and whether induction proceeds through elevation of P450IA mRNAs and occurs as a result of either transcriptional activation or message stabilization.

The EROD assay of pyridine-induced microsomal suspensions was performed to examine whether pyridine increased the expression of forms of P450 (P450IA1 and P450IA2) known to be induced by PAH and to be active in carcinogen metabolism. The results of this study revealed that pyridine treatment increased microsomal EROD activity in a time-dependent manner and suggested that P450IA1 was induced, with a maximal increase in activity being noted from ~10 to 16 hr after treatment. Although both P450IA1 and P450IA2 can contribute to the microsomal EROD activity, P450IA1 has been shown to be ~10-fold more active than P450IA2 (19). These metabolic data are supported by the results of immunoblot analyses, which showed that P450IA1 protein was increased between ~10 and 16 hr and decreased to levels present in untreated animals at 48 hr.

The time course for achieving maximal P450IA1 mRNA levels after pyridine treatment was more rapid, reaching a peak between 5 and 12 hr, than the time course of P450IA1 mRNA elevation reported for 3-MC or β-naphthoflavone, where peak P450IA1 mRNA levels were recorded ~15–17 hr after treatment (20, 22). This may reflect a different cellular response to pyridine or differences in pharmacokinetics. A specific oligonucleotide probe recognizing P450IA2 mRNA was also used to assess P450IA2 mRNA levels after a single dose of pyridine. Unlike 3-MC or β-naphthoflavone, which cause a 3–10-fold or greater elevation of P450IA2 mRNA, pyridine produced only a ~1.5–2-fold increase in P450IA2 mRNA levels at 5 and 12 hr, with the slightly greater increase occurring at 12 hr. Thus, the elevation and decline of P450IA1 and P450IA2 mRNA levels appear to occur in parallel, as has been reported for PAH.

Pyridine is clearly a less potent and less efficacious inducer of P450IA1 and P450IA2 than is 3-MC, as exemplified by comparison of the relative increase in either P450IA1 or P450IA2 protein levels at 24 hr after 3-MC administration or P450IA1 and P450IA2 mRNA levels at 5 hr after treatment with 3-MC. Pyridine, however, does appear to be a more potent and efficacious inducer than the food mutagens 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline and 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine, which, at 50 mg/kg (intraperitoneally) for 3 days, have been shown to elevate P450IA1 and P450IA2 protein levels, albeit without detectable increases in mRNA levels (23, 24).

The increase in the P450IA1 and P450IA2 mRNA levels may be the result of either transcriptional activation or mRNA stabilization. Although it is tempting to suggest transcriptional activation as the molecular mechanism for induction, it is necessary to conduct further experiments to either confirm or eliminate this mechanism, versus that of mRNA stabilization. Nonetheless, the potential for transcriptional activation of the P450IA1 gene is significant, because in untreated animals the level of P450IA1 mRNA is extremely low and is undetectable by normal hybridization analyses. This is supported further by the current results obtained from Northern blot analysis and PCR.

PCR was also used in a multiplex assay system to examine the increase in P450IA1 mRNA and P450IA2 mRNA relative to P450IIE1 mRNA, which is not induced by transcriptional activation. Although the sensitivity of this technique precludes quantitation of data at this juncture, it is apparent from these results that P450IA1 mRNA and, to a somewhat lesser extent, P450IA2 mRNA levels are elevated after pyridine treatment. Thus, both slot and Northern blot analyses, as well as PCR, provide evidence for induction of P450IA1 and P450IA2 mRNA. Moreover, the multiplex PCR assay demonstrates the feasibility of using this approach to detect and monitor simultaneously, and rapidly, the level of mRNAs associated with several different P450s.

These data show that pyridine induces the P450IA gene subfamily, with pronounced increases in enzymatic activity, protein and mRNA levels. Furthermore, these data illustrate that this amphipathic solvent, which is widely used in industry and is present in tobacco smoke, rapidly modulates the expression of different P450 gene families by separate mechanisms. The forms of P450 (P450IA1, P450IA2, and P450IIE1) that are induced, simultaneously and relatively rapidly, by this solvent are those that are primarily active in carcinogen metabolism, a profound metabolic effect for a small molecule such as pyridine.

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