Oxidation of Indole by Cytochrome P450 Enzymes[†]

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ABSTRACT: Indole is a product of tryptophan catabolism by gut bacteria and is absorbed into the body in substantial amounts. The compound is known to be oxidized to indoxyl and excreted in urine as indoxyl (3-hydroxyindole) sulfate. Further oxidation and dimerization of indoxyl leads to the formation of indigoid pigments. We report the definitive identification of the pigments indigo and indirubin as products of human cytochrome P450 (P450)-catalyzed metabolism of indole by visible, ^{1}H NMR, and mass spectrometry. P450 2A6 was most active in the formation of these two pigments, followed by P450s 2C19 and 2E1. Additional products of indole metabolism were characterized by HPLC/UV and mass spectrometry. Indoxyl (3-hydroxyindole) was observed as a transient product of P450 2A6-mediated metabolism; isatin, 6-hydroxyindole, and dioxindole accumulated at low levels. Oxindole was the predominant product formed by P450s 2A6, 2E1, and 2C19 and was not transformed further. A stable end product was assigned the structure 6*H*-oxazolo[3,2-*a*:4,5-*b*′]diindole by UV, ^{1}H NMR, and mass spectrometry, and we conclude that P450s can catalyze the oxidative coupling of indoles to form this dimeric conjugate. On the basis of these results, we propose that the P450/NADPH-P450 reductase system can catalyze oxidation of indole to a variety of products.

Indigo is considered to be the oldest dye, with uses known in ancient times (*1*, *2*). The dye was generally extracted from various species of plants, where it occurs as a glucoside precursor (indoxy-β-D-glucoside, "plant indican") that can be converted to indigo by hydrolysis and oxidation. The first chemical synthesis in 1878 (*3*) and the first commercial process in 1890 (*4*) led to the replacement of indigo production from the natural source by the commercial synthesis. More recently, bacterial systems have been used for commercial indigo production. Several microbial oxygenases are known to oxidize indole to indoxyl (3-hydroxyindole), which rapidly oxidizes in air and dimerizes to form indigo in a base-catalyzed reaction (*5*). Currently, a bacterial system is used commercially in which *Escherichia coli* has been engineered to express a tryptophanase to produce more indole and a naphthalene (di)oxygenase to convert indole to indoxyl, which subsequently oxidizes spontaneously to generate indigo (*2*, *6*). A pink pigment, indirubin, is a common byproduct of indigo synthesis, generated by the reaction of indoxyl with isatin (indole-2,3-dione), an oxidation product of indoxyl. An isatin hydrolase is coexpressed in the industrial bacterial system to control the level of isatin and thus reduce the concentration of indirubin in commercial indigo preparations (*7*).

The dyes indigo and indirubin have also been found in human urine (*8*−*10*). It is generally believed that tryptophan is degraded by intestinal bacteria to indole, which is then absorbed and metabolized within the liver to indol-3-yl sulfate ("metabolic indican") and then excreted in the urine (*10*, *11*). The sulfate ester is then hydrolyzed by bacteria in the urine, and the indoxyl thus formed undergoes spontaneous oxidation and dimerization to form indigo and indirubin. Indican is a well-known component of urine (*10*, *11*), and indicanuria has been linked to various conditions (*12*−*14*). Various studies have supported the individual steps in this pathway, including the hydroxylation of indole in mammalian liver (*15*), but the enzymes responsible for hydroxylation of indole and its subsequent sulfonation have not been identified.

P450[1] enzymes are found throughout the phylogenetic spectrum, from Archebacteria to humans (*17*). Most species, including humans, contain multiple genes of this superfamily

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[1] Abbreviations: P450, cytochrome P450 [also termed heme thiolate protein 450 (*16*)]; NPR, NADPH-P450 reductase (hNPR denotes human); PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactoside; TB, terrific broth; MS, mass spectrometry; APCI, atmospheric pressure chemical ionization; CID, collision-induced dissociation; oxazolodiindole, 6*H*-oxazolo[3,2-*a*:4,5-*b*′]diindole; Me_2-SO, dimethyl sulfoxide; $NaDodSO_4$, sodium dodecyl sulfate.

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(*18*). Some P450s are critical to normal mammalian physiology (*19*). The levels of some of the others that have been characterized, principally as xenobiotic-metabolizing enzymes, vary considerably. This information, plus results of studies with transgenic deletions in mice, argues that the functions of these P450s may not be well-defined or critical in the absence of exposure to chemicals that can produce a dangerous response (*20*−*22*).

Recently, we found that when some recombinant human P450 enzymes are functionally coexpressed with their natural redox partner NPR in *E. coli*, bacterial cultures spontaneously turn a dark blue color on induction of P450 expression (*23*). We considered the hypothesis that the blue color might be indigo. Preliminary work with human P450s 2A6 and 2E1 indicated that a pigment with similar spectral and chromatographic properties to indigo could be isolated from culture media (*23*).

The objectives of the current study were to definitively identify the pigments and related indole metabolites formed in bacterial cultures containing a functional recombinant P450 monooxygenase system, to determine the abilities of different human P450s to catalyze indigo formation, and to establish the chemical mechanisms underlying this process.

EXPERIMENTAL PROCEDURES

Chemicals. Indole, isatin, and oxindole were obtained from Aldrich Chemical Co. (Milwaukee, WI). 4-Hydroxyindole, 5-hydroxyindole, 3-acetoxyindole (indoxyl acetate), and porcine liver esterase were from Sigma Chemical Co. (St. Louis, MO). 3-Methylindole was purchased from Riedel-de Haen (Seelze, Germany). Dioxindole was prepared by $Na_2S_2O_4$ reduction of isatin (*24*) and identified by its characteristic UV spectrum (following HPLC).

Spectroscopy. UV−visible spectra were recorded in $CHCl_3$ using a Cary 14/OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA). NMR spectra were recorded in d_6-Me_2SO at 298 K using Bruker AM-400 and AM-500 instruments (Bruker, Billerica, MA) in the Vanderbilt facility. Mass analysis was carried out using a Finnigan TSQ7000 triple-stage instrument (Finnigan, Sunnyvale, CA) interfaced with a direct infusion system by an APCI source. Infusion was performed at a flow of 100 μL min^{-1} of sample solution in H_2O/CH_3OH (50/50, v/v) containing 0.1% CH_3CO_2H (v/v). N_2 was used as the sheath gas (70 psi) and auxiliary gas (10 psi). The heated capillary was maintained at 195 °C. CID was performed using Ar at a pressure of 2.3 mTorr at a collision offset of −28.0 V.

Bacterial Cultures. Bicistronic plasmids for expression of P450s 1A1, 1A2, 2A6, 2C9 (wild type), 2C19, 2E1, 3A4, 3A5, and 3A7 and NPR were constructed as described previously (*23*, *25*−*27*). Bicistronic vectors for P450 2B6 and wild-type P450 2D6 were prepared using similar methods.

Expression was performed in *E. coli* using TB media fortified with trace elements, thiamine, δ-aminolevulinic acid, ampicillin, and IPTG according to established protocols (*28*, *29*). Bacterial membranes were prepared and incubated as described (*25*, *28*).

Microsomal Preparations. Microsomes were prepared either from livers of untreated male Wistar rats (150−200 g) or human liver samples (designated "HL" and a sample number, obtained according to protocols approved by The University of Queensland and affiliated hospital institutional ethics committees or from Tennessee Donor Services, Nashville, TN).

Enzymes. P450 2A6 was modified from a published bacterial expression system (*30*) by the addition of a C-terminal $(His)_5$ tag and purified using Ni^{2+}-nitrilotriacetate affinity chromatography by C. Rizzo, Vanderbilt University (unpublished results). Rat NPR was expressed in bacteria and purified as described (*31*, *32*).

Isolation and Characterization of Pigments Formed in Bacterial Cells. A 1-L culture of P450 2A6 was grown for 48 h at 29 °C using the general conditions described above (*23*). Centrifugation (at 1200*g* for 1 min) yielded a dark blue pellet, which was collected and washed five times with H_2O (suspension and recentrifugation each time). The material was suspended in 10 mL of *N*,*N*-dimethylformamide and subjected to repeated sonication with a microprobe. $CHCl_3$ (100 mL) was added, and the solution was washed five times with an equal volume of H_2O. The $CHCl_3$ layer was dried with Na_2SO_4 and filtered through paper (A_{600} = 0.46, volume 156 mL). The material was concentrated at 50 °C in vacuo to a small volume, which was streaked on a 1 mm × 20 cm × 20 cm silica gel G TLC plate. The plate was developed with $CHCl_3/CH_3OH$/50:1, v/v. Individual colored bands were excised and extracted with $CHCl_3/CH_3OH$ (1:1, v/v) and then with acetone, followed by centrifugation and concentration of the combined organic extracts under N_2 (*23*).

Yields of indigo produced in bacterial cultures were estimated as described previously (*2*). Briefly, P450 2A6/hNPR cultures were incubated under standard conditions. Cultures were sampled at various intervals up to 120 h. Aliquots of culture medium were extracted twice with ethyl acetate; A_{600} was measured, and indigo concentrations were estimated with reference to a standard curve of synthetic indigo dissolved in ethyl acetate.

Assay of Blue Pigment Production in Vitro. Bacterial membranes containing recombinant P450 enzymes and hNPR (0.2 μM P450) or human or rat liver microsomes (0.5 μM P450) were incubated with 5 mM indole in the presence of 0.10 M Tris-HCl buffer (pH 7.4) and an NADPH-generating system containing (final concentrations) 1.0 mM $NADP^+$, 2.5 mM glucose 6-phosphate, and 0.5 U of glucose 6-phosphate dehydrogenase mL^{-1} (*33*). Bacterial membranes from cells transformed with the empty pCW vector or expressing recombinant hNPR alone were also included as P450-deficient controls at protein concentrations approximating the average of the protein concentrations in recombinant P450 incubations. Reactions were stopped at 0 (t = 0 controls) or 60 min by the addition of an equal volume of 1.0% $NaDodSO_4$ (w/v), and absorbance was measured at 665 nm, determined in preliminary studies to be the wavelength maximum for indigo dissolved in 0.5% $NaDodSO_4$ (w/v).

For analytical TLC, bacterial membranes containing recombinant human P450 [0.2 μM, except P450 2D6 (0.05 μM) and P450 1A1 (0.1 μM)] and NPR and human liver microsomes (0.2 μM P450) were prepared and incubated with indole (5 mM) as described above, except that after the 120-min incubation period aliquots were evaporated at room temperature. Samples were subsequently resuspended in 25 μL of Me_2SO, mixed with a vortex device, and centrifuged at 500*g* for 30 s in a microcentrifuge; aliquots of the

supernatants were removed and loaded on a silica gel 60 TLC plate, which was developed with $CHCl_3$.

Identification of Indole Products Generated in Vitro by Recombinant P450s and Liver Microsomes. Indole (5 mM) was incubated with *E. coli* membranes containing P450 2A6 and hNPR for varying time periods (5−30 min), and the products were separated by HPLC. Analysis was done with a Zorbax 6.2 × 80 mm octadecylsilane column (5 μm, MacMod, Chadds Ford, PA), using gradients composed of solvent A (20 mM potassium phosphate buffer, pH 7.0) and solvent B (CH_3CN/H_2O, 9/1, v/v). 0−15 min: 90% A, 10% B; 15−30 min: program from 10 to 50% B; 30−40 min: hold at 50% B; 40−45 min: program from 50 to 10% B. UV spectra were recorded online using a Thermo-Separations UV6000 rapid scanning spectrophotometer (ThermoSeparations, Piscataway, NJ) (scanning from 200 to 320 nm) to characterize the oxidation products. Further characterization of products was done by HPLC/MS analysis of the samples (vide supra), with comparisons made of the A_{240} traces (online) with the profiles obtained using rapid-scanning UV analysis. In the case of products of indole oxidation, compounds were introduced from a 2.1 × 150 mm Zorbax octylsilane HPLC column with a gradient of CH_3OH increasing from 4.5 to 81% over 32 min in 0.10% aqueous HCO_2H (v/v). Under these conditions, the substrate indole was eluted at t_R 29 min (identified by co-chromatography with an external standard, UV spectra, mass spectrum: MH^+ at *m*/*z* 118).

To compare products formed from indole by different P450s, bacterial membranes (containing recombinant P450 enzymes and hNPR) and rat and human liver microsomes (0.2 μM P450) were incubated with 5 mM indole under conditions supporting P450 catalysis (0.10 M Tris-HCl, pH 7.4, and NADPH-generating system). Aliquots (500 μL) were taken at zero and 120 min, and 3-methylindole (10 μg) was added prior to extraction as an internal standard. Products formed were extracted with methyl *tert*-butyl ether, dried under air, dissolved in a 15% CH_3CN: 85% 50 mM Tris-HCl (pH 7.4) mixture (v/v), and injected onto a 3.9 × 150 mm Waters octylsilane C8 Symmetry reversed-phase HPLC column. The column was eluted at a flow rate of 1 mL min^{-1} with the following gradient. 0−15 min: 15% CH_3CN, 85% 50 mM potassium phosphate, pH 7.4; 15−30 min: 15 to 40% CH_3CN gradient; 30−40 min: 40 to 50% CH_3CN gradient; 40 to 45 min: 50 to 15% CH_3CN gradient; 45−55 min: isocratic with 15% CH_3CN (all v/v). Under these conditions isatin eluted at t_R 7.5 min and indole at t_R 36.7 min. Other peaks that were subsequently identified by mass spectrometry (vide infra) as dioxindole and oxindole eluted at 4.3 and 10.4 min, respectively. Additional minor product peaks eluting were also observed in incubations containing different P450 enzymes but were not identified.

RESULTS

Characterization of Pigments. The extracellular pigment produced in the P450 2A6 culture was separated into four components, two blue and two pink/purple (**1**−**4**, see Supporting Information). In some extracts of P450s 2A6 and 2E1, bands **2** and **4** were much more predominant. The visible spectra of components **2** and **4** (Figure 1, λ_{max} 603, 552 nm respectively) match the literature for indirubin and indigo, respectively (*8*, *9*, *34*). Much of the highly insoluble indigo also remained at the origin of the chromatogram. MS yielded an apparent MH^+ ion at *m*/*z* 263 for compounds **1**, **2**, and **4**, indicating a molecular mass of 262 (Supporting Information). Fragmentation of the *m*/*z* 263 ion in each case yielded *m*/*z* 235 (-CO), 219 (-CO, $-NH_2$), 217, and 190. The same ions were seen when standard indigo was fragmented in the same way. A definitive MH^+ for compound **3** was not apparent, and the visible spectra did not match those of compounds **2** or **4** (Figure 1). The UV−visible spectrum of compound **1** was very similar to that of **4**, but we were unsuccessful in obtaining definitive NMR and mass spectra.

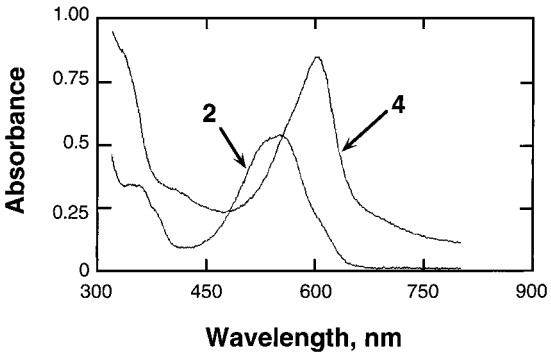


FIGURE 1: UV-visible spectroscopy of pigments **2** and **4** formed by recombinant P450 2A6 in *E. coli* cells. The pigments (**1**−**4**) were separated by TLC as described, and spectra were recorded in $CHCl_3$. The spectrum of pigment **2** is characteristic of indirubin; pigment **4** corresponds to indigo.

^1H NMR of compounds **2** and **4** provided further support for their respective assignments as indirubin and indigo (*35*), particularly the 2-dimensional spectra (Supporting Information). The symmetry of the indigo structure was observed, and indirubin showed the expected asymmetry.

Indigo production peaked between 26 and 38 h at approximately 10 mg (L of culture)$^{-1}$; however, residual blue pigment was observed in the bacterial cell pellets remaining after solvent extraction, suggesting that yields were underestimated using this procedure.

Formation of Blue Pigment in Vitro. Pigment production was seen with a range of recombinant P450 preparations (Figure 2). The P450s most efficient in the production of the blue pigment comigrating with indigo on TLC were 2A6 > 2C19 > 2E1 (Supporting Information); however, lower amounts of other pigments, particularly indirubin, were seen with most forms.

Identification of Products Generated in Vitro by Recombinant P450 2A6 and Liver Microsomes. Various indole products were generated by bacterial membranes in which both P450 2A6 and hNPR were coexpressed (Supporting Information). No metabolites were observed in the complete system that had been quenched with organic solvent immediately after the addition of NADPH to initiate the reaction. An HPLC profile very similar to that observed with membranes containing expressed P450 2A6 and hNPR was obtained with purified P450 2A6 (Figure 3, panel A). Only minimal amounts of the products were seen in membranes prepared from bacteria in which only hNPR was expressed or with purified NPR, in the absence of any P450 (Figure 3, panel B).

Under the HPLC conditions used for identification of products of indole metabolism, the substrate indole eluted

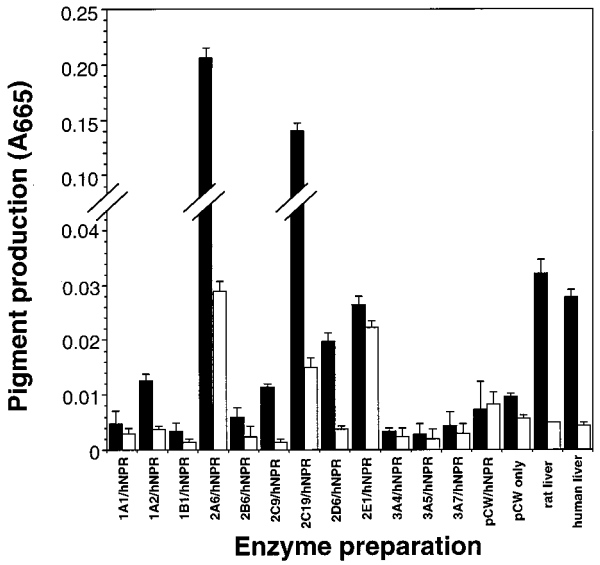


FIGURE 2: In vitro formation of blue pigments by bacterial membranes containing recombinant human cytochrome P450/hNPR or liver microsomes. Each P450 form is indicated. For P450 2C9, the wild-type allelic variant was used [(*36*); 2C9*1, encoding a protein with Arg at position 144, Tyr at 358, Ile at 359, and Gly at 417]. For P450 2D6, the variant used encoded Val at position 374 (wild type). Rat and human (HL 16 sample) liver microsomal samples are also included. In all cases, 5 mM indole was incubated with the membranes (0.2 μM P450) at 37 °C for 60 min (■), and dye production was analyzed by measuring A_{665}. Control incubations were stopped immediately after initiation ($t = 0$ control) (□) (reflects some residual indigo in membranes). The differences between $t = 0$ and $t = 60$ min were significant ($p < 0.05$) and $t = 60$ values more than twice the background $t = 0$ level for P450s 1A2, 2A6, 2C9, 2C19, and 2D6 and the liver microsomes. Color formation was also significant for P450 2E1 ($p < 0.05$) but $t = 60$ levels were less than twice background as was also the case for the pCW control. For P450s 2E1, 2C19, and 2A6, the elevated background pigment reflects indigo produced during culture and carried through in membrane preparation. The levels of apparent color formation seen in pCW and pCW/hNPR controls reflect the level of sensitivity of this microtiter plate assay.

at t_R 35 min (identified by UV spectrum, co-chromatography with an external standard, and mass spectrum: MH^+ at *m*/*z* 118). Five of the product peaks eluted earlier and were identified by their UV and mass spectra and literature comparisons (see UV spectra in Supporting Information):

Dioxindole. t_R 3.6 min, UV λ_{max} 210, 254, 295, and spectrum identical to those published by Ward (*37*) and Conforth et al. (*38*). The product generated by $Na_2S_2O_4$ reduction of isatin (*24*) yielded the same compound, as judged by HPLC t_R and UV spectra.

Isatin. t_R 7.1 min, UV λ_{max} 244, 305. t_R and spectrum identical to the commercial product (*37*, *39*). MS: *m*/*z* 148 (MH^+).

Oxindole. t_R 9.5 min, UV λ_{max} 203, 248, both the same as the commercial material (*38*, *40*). MS: *m*/*z* 134 (MH^+). ^1H NMR ($CDCl_3$) δ 3.56 (s, 2H, H-1), 6.88 (d, 1H, H-4 or H-7), 7.04 (t, 1H, H-5 or H-6), 7.24 (t, 1H, H-5 or H-6), 7.27 (d, 1H, H-4 or H-7), 7.53 (broad, 1H, NH).

Indoxyl. t_R 13.4 min, UV λ_{max} 239 nm. t_R and UV spectra identical to material generated by reaction of commercial 3-acetoxyindole and hog liver esterase. MS: *m*/*z* 134 (MH^+). This product was seen only in short incubations (≤15 min).

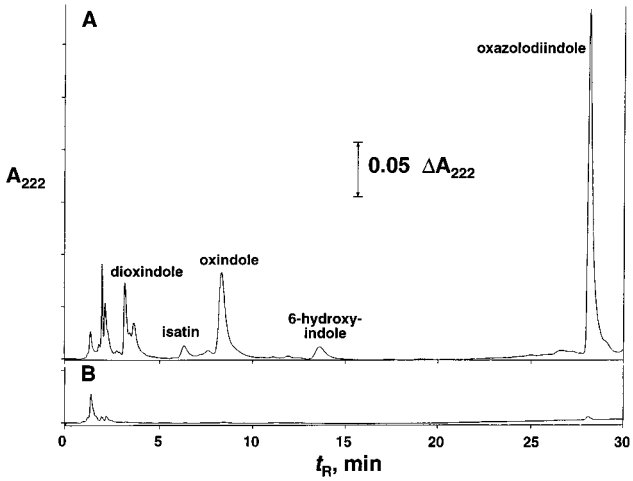


FIGURE 3: HPLC of extracts of incubations. All incubations were done in 0.10 M Tris-HCl buffer (pH 7.4) with 5 mM indole for 30 min unless indicated otherwise (1.0 mL volume) in the presence of an NADPH-generating system (*33*). One-fourth of the methyl *tert*-butyl ether extract was analyzed. (A) The system included 0.4 μM P450 2A6, 0.4 μM rat NPR, and 30 μM L-α-dilauroyl-*sn*-glycero-3-phosphocholine. (B) *E. coli* membranes containing hNPR (1.2 μM). Identities of the indicated peaks were confirmed by online UV spectral analysis (Supporting Information).

6-Hydroxyindole. t_R 14.8 min, UV λ_{max} 218, 293. Spectrum identical to that in the literature (*41*, *42*). MS: *m*/*z* 134 (MH^+).

6*H*-Oxazolo[3,2-*a*:4,5-*b*′]diindole is the structure assigned to the compound eluting at t_R 28 min (Figure 3, panel A). The mass spectrum (Figure 4, panel A) indicated a strong *m*/*z* 247, assigned as MH^+. A possible molecular formula is $C_{16}H_{10}N_2O$ (M_r 246). One structure cited in SciFinder (American Chemical Society) is that of 6*H*-oxazolo[3,2-*a*:4,5-*b*′]diindole (Reg. no. 97890-29-4, no references cited). The UV spectrum (Figure 4, panel B) was similar to that of indole and no significant visible spectrum was observed, indicating a lack of extended conjugation, so the compound cannot be "indoxyl red," 2-indol-3-yl-3*H*-indol-3-one (*43*, *44*). The amount of material isolated permitted acquisition of an ^1H NMR spectrum (Figure 4, panel C) but not decoupling or a 2-dimensional spectrum. The assignments were made as indicated in the inset of Figure 4, panel C. The compound could not be produced (as judged by HPLC t_R and UV spectrum) by treating 3-acetoxyindole with hog liver esterase, whether an equimolar amount of indole was present in the incubation.

No peaks produced by recombinant P450 2A6 or human liver microsomes corresponded to 4- or 5-hydroxyindole (commercial samples), as judged by t_R and UV spectra.

Characteristics of Indole Metabolism by P450s. Several human P450s were coexpressed with human NPR in *E. coli* membranes and used to analyze indole metabolism (P450s 1A1, 1A2, 1B1, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7). The amounts of indole products appeared to be highest with P450s 2A6 and 2C19 (Figure 5, panels A and B), followed by P450 2E1 (Figure 5, panel C) and P450 2D6 (not shown). (For a typical P450-deficient control experiment see Figure 3, panel B.) As indicated in Figure 5, the profiles of products were similar but not identical. The other P450s produced smaller amounts of peaks that migrated at similar t_R values, but the identities of these compounds were not further confirmed by spectroscopy.[2]

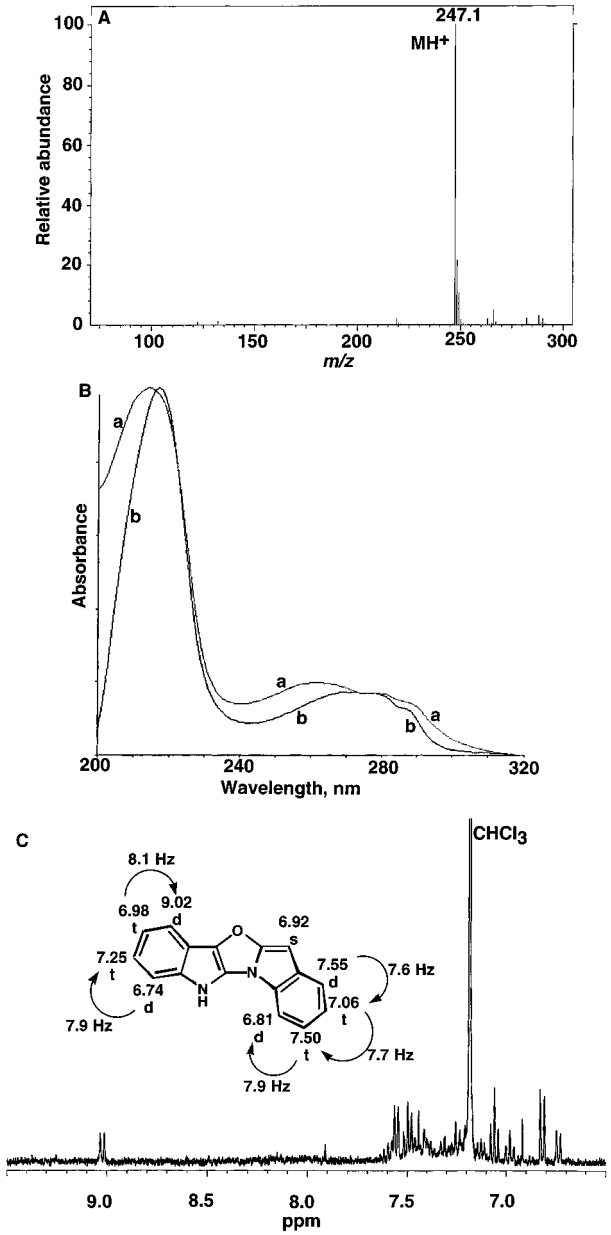


FIGURE 4: Spectral characterization of t_R 29 min peak (Figure 6) as 6*H*-oxazolo[3,2-*a*:4,5-b′]diindole. (A) Mass spectrum (APCI). (B) (a) UV spectrum [online from HPLC, in 50 mM potassium phosphate (pH 7.0)/H_2O/40% CH_3CN, v/v]; (b) UV spectrum of indole. (C) ^1H NMR ($CDCl_3$, 500 MHz). Shift (δ) and pattern assignments are shown, and *J* values (in Hz) are indicated with arrows.

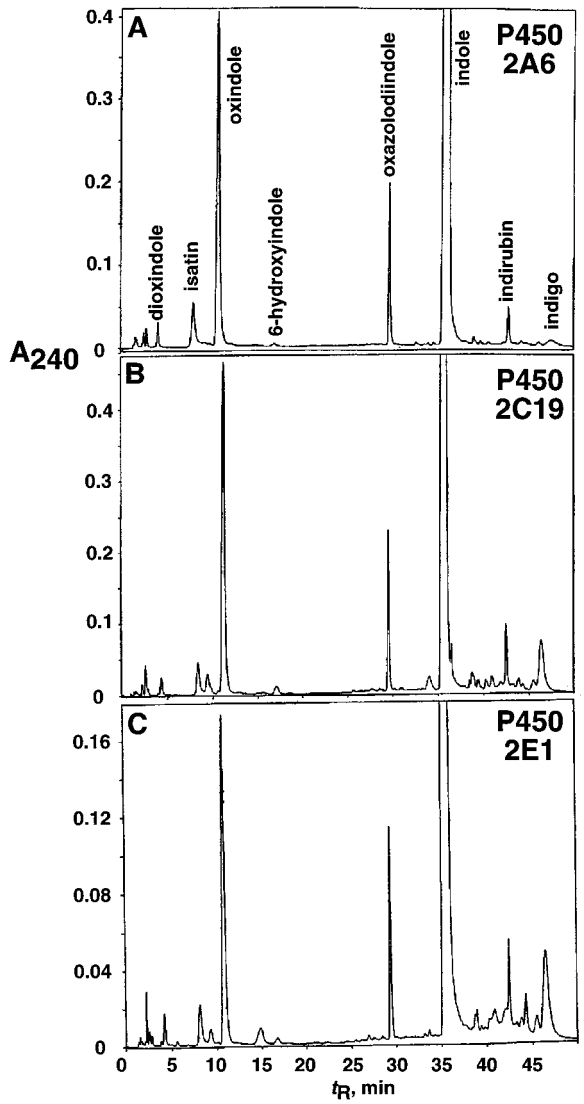


FIGURE 5: HPLC of indole metabolites produced by bacterial membranes containing recombinant P450s and hNPR. Incubations were done for 2 h at 37 °C with (A) P450 2A6 (0.10 μM P450), (B) P450 2C19 (0.20 μM P450), or (C) P450 2E1 (0.20 μM P450).

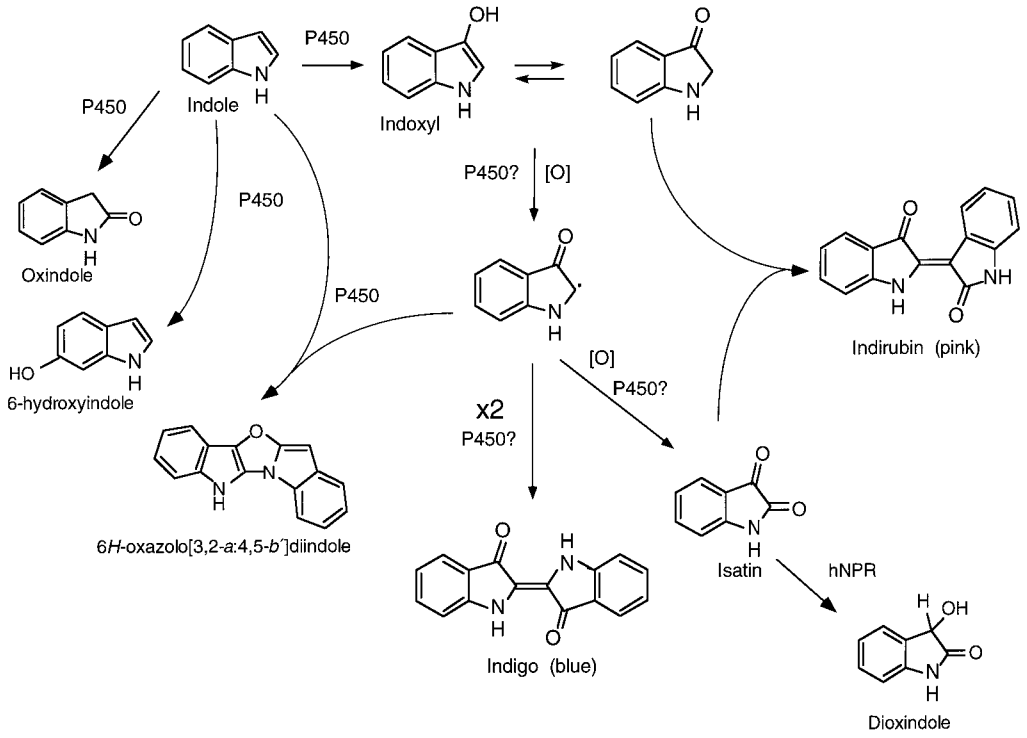
The time course for production of the major products from indole by P450 2A6 is shown in Figure 6. At each indicated time point, a reaction aliquot was withdrawn and extracted into CH_2Cl_2 to stop the reaction. Products (CH_2Cl_2 phase) were concentrated and analyzed by HPLC (see Figure 6 legend for estimation of products with extinction coefficients available). Indoxyl was detected in the early portion of the experiment (1−2 min, not shown). The production of indigo, as estimated by A_{600} in the combined CH_2Cl_2 extract and a fraction prepared from the residual aqueous phase by adding 2 vol of *N*,*N*-dimethylformamide, showed a lag but began within 2 min. The time courses for indoxyl and indigo are concluded to be related.

Oxindole appears to be the major primary oxidation product of indole formed by P450 2A6. The concentration of indole was varied in 2-min incubations with membranes containing P450 2A6 and hNPR. The parameters $k_{cat} = 6.9$ ($\pm$ 0.6) min^{-1} and $K_m = 122$ ($\pm$ 30) μM were estimated. Oxindole (1 mM) was rather stable when added to the typical P450 2A6/hNPR membrane system; no significant formation of any products was observed.

When isatin was used as the substrate in similar incubations, it was rapidly reduced to dioxindole in the P450 2A6 system (only product). Subsequent experiments with purified hNPR showed that this enzyme catalyzed the reduction efficiently itself and the P450 2A6 did not seem to contribute. The rate of reduction measured (with 1.0 mM isatin) was 290 min^{-1}.

[2] The microsomal patterns were also similar. For instance, a 2-h incubation with rat liver microsomes (0.1 nmol of P450) yielded 2.6 nmol of dioxindole, 32 nmol of isatin, 42 nmol of oxindole, 0.4 nmol of 6-hydroxyindole, and 27 nmol of oxazolodiindole, based on the assumption that ϵ_{220} for oxazolodiindole = 2× that of indole (Figure 4, panel B). A similar incubation with one human liver microsomal sample produced <1 nmol of dioxindole, 9 of nmol isatin, 51 nmol of oxindole, 1.2 nmol of 6-hydroxyindole, and 20 nmol of oxazolodiindole.

Scheme 1: Postulated Major Reactions in P450-Catalyzed Pigment Formation



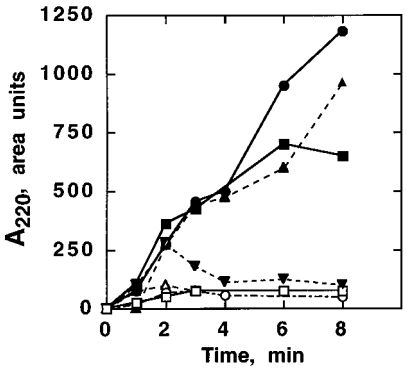


FIGURE 6: Time course of various indole products in incubations with recombinant P450 2A6/hNPR in bacterial membranes. Experiments were performed as described in Experimental Procedures with 1.0 mM indole and 0.5 μM P450 2A6. A_{600} (▲) indicates pigment production (predominantly blue due to indigo). t_R 3.3 (▼) indicates an unidentified product from HPLC. Other products are as indicated on the figure. The data points at $t = 2$ min correspond to 7 nmol of oxindole (■), 0.38 nmol of isatin (□), 0.95 nmol of dioxindole (△), and 0.14 nmol of 6-hydroxyindole (○), calculated using extinction coefficients from refs *37* and *39*−*42*. The other A_{220} integral shown is for oxazolodiindole (●).

On the basis of these results, we conclude that indole metabolism in P450 2A6/hNPR/NADPH systems and in NADPH-fortified human and rat liver microsomes follows the course shown in Scheme 1.

DISCUSSION

Observations from our laboratories have suggested that indigo (*23*) and indirubin are produced in situ in bacterial cultures in which recombinant human P450s are functionally coexpressed with their natural redox partner, hNPR. In the current work, we have definitively identified the major pigments produced in this system as indigo and indirubin by mass spectrometry and NMR. Recombinant human P450s were capable of generating indigo and indirubin in vitro, with human P450s 2A6 and 2C19 showing the highest activity. These forms also yielded blue cultures most frequently, along with P450 2E1 (*23*). Analytical TLC showed that the ratio of the pigments differed between forms, a point that may be related principally to differential rates of indoxyl production. However, the ability of hNPR-catalyzed dioxindole production to clear isatin, in a manner analogous to the isatin hydroxylase used in the commercial biotechnological method for indigo production, may also influence this ratio, since the reductase/P450 ratio varies between forms in the bicistronic system.

The structure 6*H*-oxazolo[3,2-*a*:4,5-*b*′]diindole was assigned to one of the prominent oxidation products formed by P450s (Figures 3 and 5) and is based on the mass and ^{1}H NMR spectra (Figure 4). The lack of bond conjugation is consistent with the lack of a visible electronic spectrum and would be consistent with the UV spectrum, which resembles that of indole (Figure 4, panel B). When indoxyl was generated (with 3-acetoxyindole and esterase) in the absence or presence of an equimolar concentration of indole (1 mM), none of this material was obtained. We propose that this product is formed by oxidative coupling within the active sites of P450s. Evidence for oxidative coupling of a product to form rings within the active sites of plant P450s has been presented by Zenk (*45*).

The results presented here suggest that recombinant human P450 enzymes might find application in the commercial production of indigo for the dye market. The yield of indigo formed in P450 2A6 cultures [~10 mg (L of culture)$^{-1}$] was in the same range as that obtained previously with naphthalene dioxygenases expressed in *E. coli* in the absence of any enhancement of tryptophanase activity to boost endogenous indole levels in cultures (*2*). Other applications can also be envisaged. Eukaryotic P450s may be useful in the development of transgenic plants, engineered to produce pigment

in specific tissues such as petals (e.g., flower crops) and fiber (e.g., cotton) (*46*, *47*). In another potential application, indigo production may be used as a rapid screening assay for examination of mutant P450 forms with altered catalytic activity. This approach is currently being applied in our laboratories for the screening of P450s generated by mutagenesis.

The enzymology underlying indole metabolism in vivo has received little attention to date. Liver microsomes were demonstrated to oxidize indole to indigo, indirubin, oxindole, *N*-formylanthranilic acid, and anthranilic acid in the presence of NADPH in earlier work (*15*); however, the enzymes catalyzing the hydroxylation of indole and its subsequent sulfonation have not been identified to date. Our results suggest that P450 forms in the intestinal mucosa and/or liver may subserve a role in hydroxylation of indole. Several forms oxidized indole to at least some extent suggesting that there may be considerable redundancy in the system for metabolic clearance of indole. We have been able to demonstrate the production of at least six products of indole (Figures 3 and 4): dioxindole, isatin, indoxyl, oxindole, 6-hydroxyindole, and oxazolodiindole. Although other metabolites were not identified in the current work, these may include such derivatives as 5-hydroxyoxindole, which was reported to be formed from oxindole in rat liver preparations (*15*).

Further work in our laboratories is directed toward identifying other metabolites and investigating the in vivo relevance of these biotransformation reactions and the significance of the metabolic products.

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SUPPORTING INFORMATION AVAILABLE

Figures showing TLC separation of pigments (**1**−**4**) formed by recombinant P450 2A6, mass spectra of indigo and indirubin produced by P450 2A6, 1- and 2-dimensional ^{1}H NMR spectra of indigo and indirubin produced by P450 2A6, TLC analysis of indigo and indirubin formed by several forms of P450 and microsomes, HPLC of pigments formed from incubation of indole with bacterial membranes containing P450 2A6, and UV spectra of individual products of indole metabolism by P450 2A6. This information is available free of charge via the Internet at http://pubs.acs.org.

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