Structural Gene Products of the Ah Locus

GENETIC AND IMMUNOCHEMICAL EVIDENCE FOR TWO FORMS OF MOUSE LIVER CYTOCHROME P-450 INDUCED BY 3-METHYLCOLANTHRENE*

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With the use of heterogenic stock (HS) mice, more than 9-fold differences in hepatic microsomal 3-methylcholanthrene-inducible aryl hydrocarbon (benzo[a]pyrene) hydroxylase (EC 1.14.14.2) activity and at least 4-fold differences in 3-methylcholanthrene-inducible acetanilide 4-hydroxylase activity were found. These data strongly suggest independent genetic control of two inducible cytochrome P-450-mediated monoxygenase activities known to be associated with the murine Ah locus.

The procedure is described for isolating two inducible forms of partially separated P-450 from C57BL/6N treated with 3-methylcholanthrene, and an antibody to each of these forms was developed. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a molecular weight of about 55,000 is estimated for both partially separated forms. Limited proteolysis reveals differences in the peptide composition of these two cytochromes. P-450 is more closely associated with polycyclic aromatic compound-inducible aryl hydrocarbon hydroxylase activity and NADPH oxidation in the presence of benzo[a]pyrene, whereas P-448 is more closely associated with polycyclic aromatic compound-inducible acetanilide 4-hydroxylase activity and NADPH oxidation in the presence of acetanilide. The Soret peaks of the reduced hemoprotein CO complex are about 449.3 and 448.0 nm for P-450 and P-448, respectively. Goat anti-P-450 immunoglobulin inhibits 3-methylcholanthrene-inducible aryl hydrocarbon hydroxylase activity while not affecting 3-methylcholanthrene-inducible acetanilide 4-hydroxylase activity. Rabbit anti-P-448 immunoglobulin blocks 3-methylcholanthrene-inducible acetanilide 4-hydroxylase activity while not affecting 3-methylcholanthrene-inducible aryl hydrocarbon hydroxylase activity. Evidence is also presented for the possible presence of P-448 in the liver microsomes of the control, untreated mouse. By means of the pyridoxal phosphate/NaB[^H] method for labeling intact microsomes in vitro followed by immunoprecipitation, it was confirmed that the polycyclic aromatic compound-inducible (P-450) induction (and its associated aryl hydrocarbon hydroxylase activity) in fetal mouse liver by 2,3,7,8-tetrachlorodibenzo-p-dioxin develops earlier in gestational age than P-448 induction (and its associated acetanilide 4-hydroxylase activity) by 2,3,7,8-tetrachlorodibenzo-p-dioxin.

These data, plus studies involving inhibitors in vitro, all provide direct biochemical evidence that two structural gene products controlled by the Ah locus represent distinctly different forms of P-450. These data also show that the routinely accepted "cytochrome P-448" is not the most active form of cytochrome P-450 catalyzing polycyclic aromatic compound-inducible aryl hydrocarbon hydroxylase activity in mouse liver, in accordance with previous conclusions from this laboratory for rabbit and rat liver.

The murine Ah locus regulates the induction (by polycyclic aromatic compounds such as 3-methylcholanthrene, \(\beta\)-naphthoflavone, or TCDD\) of numerous drug-metabolizing enzyme activities in the liver and in numerous nonhepatic tissues that have been examined (reviewed in Refs. 2 and 3). The presence of a cytosolic receptor for polycyclic aromatic inducers in genetically "responsive" mice appears to be essential for the induction process (4, 5), although the presence of this receptor per se might not guarantee that the induction process will proceed (6). Genetically "nonresponsive" mice appear to have a lower number and/or poorer affinity of cytosolic binding sites (5). The cytosolic receptor is thus viewed as the major product of the regulatory gene of the Ah "cluster."

Increasing evidence (2, 3) indicates that five, and probably many more than five, structural genes are "turned on" during the sequence of events following exposure of the animal to the polycyclic aromatic inducer. Not only the induction of two or more forms\(^2\) of P-450, but also microsomal UDP glucuronosyltransferase activity and two cytosolic enzyme activities (reduced NAD(P):menadione oxidoreductase and ornithine

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Characterization of Mouse Liver P-450 and P-448

decarboxylase) appear to be induced and under the same controlling Ah gene (reviewed in Ref. 3). De novo protein synthesis of one or more forms of P-450 has been shown (9) to occur in genetically responsive mice following β-naphthoflavone treatment. Indirect electrophoretic, spectral, and catalytic data with intact liver microsomes suggested that cytochromes P-450 and P-448 possess differing molecular weights and are under different temporal control; P-450 appears to be inducible several weeks earlier in development than P-448 in 3-methylcholanthrene-treated rabbits (10). More recently, similar evidence in rat and mouse liver shows a small difference in temporal control of the expression of these two structurally similar products, P-450 and P-448 appearing to be inducible several days earlier in development than P-448 in TCDD-treated animals (13). In this report we demonstrate the partial separation of these two polycyclic hydrocarbon-inducible forms of P-450 from mouse liver. The spectral and catalytic data with these partially separated cytochromes and in experiments with antibodies to these proteins provide direct evidence in support of our earlier postulation, i.e., that induced aryl hydrocarbon hydroxylase activity and the routinely accepted “cytochrome P-448” are not identical, in mouse, rat, or rabbit liver.

EXPERIMENTAL PROCEDURES

Materials—Benzo[a]pyrene, 3-methylcholanthrene, benzo[a]lanthracene, Triton X-100, dithiothreitol, dialuroyl-3,14-l-lactilin, NADPH, NADH, ethylene diaminetetraacetic acid, and pyridoxal phosphate were purchased from Sigma (St. Louis, MO); cholic acid, α-naphthoflavone, and octyl-diamine-1,8 from Aldrich Chemical Co. (Milwaukee, WI); cytochrome b₅ from Miles Laboratories (Kankakee, III); Sephrose 4B from Pharmacia Co. (Uppsala, Sweden); hydroxyapatite (Bio-Gel HT) from Bio-Rad (Richmond, CA); and complete and incomplete Freund’s adjuvants from Grand Island Biochemical Co. (Grand Island, NY). S.B₁₂ (tradename: Zwittner TM₁₁), N-tetradecylox-N,N-di methyl-3-ammonio-1-propane sulfonate was bought from Calbi- ochem (La Jolla, CA). Other materials were obtained from the sources previously cited (10). Acetanilide uniformly labeled with ¹⁴C in the ring (11 mCi/mmol) was bought from California Bionuclear Corp. (Sun Valley, CA) and generally labeled [¹⁴H]4-hydroxyacetanilide (400 mCi/mmol) was purchased from New England Nuclear Co. (Boston, MA). NADPH(¹⁴H) (7.9 Ci/mmol) was purchased from Amersham (Ar- linington Heights, IL). Benzyl cytochrome P-450, benzyl lanthanide, acetanilide, and 4-hydroxyacetanilide were recrystallized from warm benzene by the addition of cold methanol. Cholic acid was further purified by recrystallization in 50% ethanol. TCDD was a generous gift of the Agricultural Division of Dow Chemical Co. (Midland, MI). Highly purified preparations of hepatic NADPH-cytochrome P-450 reductase from untreated pigs (14) were a generous gift from Dr. Bettie Sue S. Masters (University of Texas Health Science Center at Dallas, TX). HS mice were generously provided by Dr. Allan C. Collins (University of Colorado at Boulder, CO). B6 and D2 mice were obtained from the Veterinary Resources Branch, National Institutes of Health Animal Supply (Bethesda, MD). All animals were used as weanlings of either sex, except for several pregnant B6 mice.

Treatment of Mice and Preparation of Microsomes—3-Methylcholanthrene in corn oil was administered as a single intraperitoneal dose of 250 mg/kg (15) 5 days before killing, unless otherwise indicated. TCDD in p-dioxane was administered as a single intraperitoneal dose of 25 µg/kg (15) 24 h before killing. Untreated controls received corn oil (50 ml/kg) or p-dioxane (40 µl/kg) within the same time frame. For assays of cytochrome P-450 contents from intact microsomes, the liver microsomes in 0.25 M potassium phosphate buffer, pH 7.25, containing 30% glycerol were prepared exactly as described recently in detail (10).

Purification of P-450 and P-448 from 3-Methylcholanthrene-treated B6 Liver—We decided to use the purification method of Hashimoto and Imai (15) for rabbit P-448. Minor modifications are described in detail recently by this laboratory (10). The standards, with their subunit molecular weights, included: ovalbumin, 45,000; glutamate dehydrogenase, 53,000; catalase, 58,000; and albumin, 68,000. Because of limitations in this technique, we have arbitrarily estimated the molecular weights of electrophoretic bands only to the nearest 1,000.

Peptide Analysis—Limited proteolysis of P-450 or P-448 was performed by the method of Cleveland and co workers (26). Approximately 10 µg of cytochrome was treated in 35 µl of 10 mM Tris/acetate/guanidine buffer, pH 9.0, containing 0.5% sodium dodecyl sulfate at 100°C for 2 min and then digested by variable amounts (1 µg or 2.5 µg) of S. aureus V8 protease at 37°C for 30 min. The protease was stopped by adding 10⁻³ M phenylmethylsulfonyl fluoride and 2.5% sodium dodecyl sulfate. After being brought to 100°C with β-mercaptoethanol for 2 min, the digested cytochrome fractions were examined by sodium dodecyl sulfate polyacrylamide gel (constant 13.5% acrylamide) electrophoresis.

Development of Antibody to P-450 or P-448—Highly purified P-450 was injected subcutaneously into the leg of a goat. For the first immunization, 2.0 µg of cytochrome with complete adjuvant was injected; 2 and 4 weeks later, the second and third injections, respectively, of 1.0 µg of cytochrome each time with incomplete adjuvant were given. Partially purified P-448 was injected subcutaneously into the foot pads of two rabbits. To each rabbit, 500 µg of P-448 with complete adjuvant was injected. For the first time, with incomplete adjuvant; the second injection was given with incomplete adjuvant was given for the second immunization. Immunoglobulin G fractions from preimmune goat or rabbit serum, anti-P-450 goat serum, and anti-P-448 rabbit serum were prepared by ammonium sulfate fractionation and were then dissolved in 100 mM potassium phosphate buffer, pH 7.5.

Reconstitution of Monoxygenase Activities—The reconstitution experiments were essentially as described (7). For the measurement of NADPH oxidation, the 1 mol reaction mixture contained 0.1 n mole of P-450 or P-448, 0.30 unit of NADPH-cytochrome P-450 reductase, 30 µg of dialuroyl-3,14-lactilin, 50 µmol of Hapes buffer, pH 7.4, 15 µmol of MgCl₂, and benzo[a]pyrene (600 n mole added in 90 µl of acetone) or acetanilide (21 µmol added in 10 µl of acetone). To allow for temperature corrections, 1 ml of the reaction mixture was brought to an elevated temperature by the addition of 0.2 µl of NADPH, and decreases in absorbance at 340 nm were measured; an extinction coefficient of 6.22 mM⁻¹ cm⁻¹ for NADPH was used (27). For the measurement of aryl hydrocarbon hydroxylase or acetanilide 4-hydroxylase activity, the P-450 or P-448 buffer, pH 7.25, containing 30% glycerol, 0.4% cholate, 1.0 mM dithiothreitol, and 0.05% Triton X-100 instead of Emulgol 913. Next, a hydroxyapatite column was equilibrated with 50 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol and 0.2% Triton X-100. The single major peak obtained from the 8-aminoocyli-Sepharose 4B column then eluted by stepwise increases in concentration of potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.2% Triton X-100, and 0.1 mM dithiothreitol. Selected eluted fractions were pooled separately and concentrated by small hydroxyapatite columns.

Spectrophotometry—Microsomal P-450 total content and P-450 or P-448 content of partially purified fractions were determined by the method of Omura and Sato (17). An extinction coefficient of 91 mM⁻¹ cm⁻¹ was used for the difference in absorbance between the Soret maximum around 450 nm and the baseline at 490 nm for the heme-protein. CO reduced complex. Differences in the extinction coefficient between P-450 and P-448 (if any differences exist) are probably very small. The maximal absorbance of P-450 in an extinction coefficient of 1014 mM⁻¹ cm⁻¹ was used for the difference in absorbance between 417 nm and 490 nm of the oxidized cytochrome (18,19). Drug binding difference spectrophotometry by the usual method (20) or by the tandem-cuvette technique previously described (21) was also performed with the partially separated P-450 or P-448 fractions. Wavelength measurements were performed with the use of a holmium oxide crystal (Fisher Scientific Co., Silver Spring, MD).

Enzyme Assays—Aryl hydrocarbon hydroxylase (22) and acetanilide 4-hydroxylase (23) activities were determined by the method described in the references cited. One unit is arbitrarily defined as that amount of enzyme catalyzing per min at 37°C the formation of 1 pmol of benzo[a]pyrene hydroxylated product. Specific enzyme activity units per mg of protein. Protein determination was also performed by the method of Lowry et al. (24).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—The slab gel electrophoresis method was that of Laemmli (25), as described in detail recently by this laboratory (10). The standards, with their subunit molecular weights, included: ovalbumin, 43,000; glutamate dehydrogenase, 53,000; catalase, 58,000; and albumin, 68,000. Because of limitations in this technique, we have arbitrarily estimated the molecular weights of electrophoretic bands only to the nearest 1,000.
Characterization of Mouse Liver P-450 and P-448

was incubated for 1 min at 37°C with the NADPH-cytochrome P-450 reductase and diaroyl-a-I-lecinin, in the amounts described above, following which the regular buffer and assay conditions were carried out as previously described (22, 23).

Inhibition of Monoxygenase Activities—o-Naphthoflavone (added in 20 μl or less of methanol), benzo[a]pyrene and benzo[a]-anthracene (added in 20 μl or less of acetone), and acetanilide (added in 3 min at 37°C, or less of methanol) were all studied as inhibitors. The inhibitory effects on either monoxygenase activity were examined in both intact microsomes and the reconstituted system. Each inhibitor was preincubated for 5 min at 0°C before addition of substrate and the subsequent enzyme assay at 37°C, as previously described (21).

Immunoprecipitation of 3H-labeled P-450 and P-448—The in vitro labeling of intact microsomes by pyridoxal phosphate/NaBH₄, and the immunoprecipitation of the cytochromes from 3H-labeled intact membranes were carried out by the previously reported procedures (28, 29). Liver microsomes from 3-methylcholanthrene-treated B6 meanings or from TCDD-treated B6 fetuses (estimated gestational age of 20 days) were used. In the latter case, the pregnant mother had received intraperitoneally the inducer TCDD 24 h before killing. Between 1.5 and 2.0 mg of microsomal protein was incubated with 1 μg pyridoxal phosphate in a total volume of 0.80 ml, followed by the addition of approximately 90 mCi of NaBH₄, to make a final volume of 0.725 ml (28). After in vitro labeling for 30 min at 0°C, the treated microsomes were centrifuged at 105,000 X g for 60 min at 4°C through a 0.5 M sucrose cushion containing 100 mM potassium phosphate buffer, pH 7.5, and then homogenized in 0.25 M potassium phosphate buffer (pH 7.5) containing 20% glycerol. The labeled microsomes (4 mg/ml) were solubilized in 100 mM potassium phosphate buffer, pH 7.5, containing 200 mM KCl, 20% glycerol, and 25% sodium cholate and further centrifuged at 105,000 X g for 60 min. Aliquots of the supernatant fraction (approximately 30 μl) were used for immunoprecipitations by about 1.0 mg of anti-P-450 or anti-P-448 following incubation at 4°C overnight. The immunoprecipitate was washed at 4°C three times with 100 mM potassium phosphate buffer, pH 7.5, containing 200 mM KCl, 1% sodium cholate, and 0.25% 8B₄, and then once with water to remove potassium ions. The immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described above.

RESULTS

Genetic Differences in Aryl Hydrocarbon Hydroxylase and Acetanilide 4-Hydroxylase Inducibility among HS Mice—We decided to look at the most outbred population of mice available. In Fig. 1, an approximate correlation can be seen (i.e. the norm of higher inducible aryl hydrocarbon hydroxylase activity in mice having higher acetanilide 4-hydroxylase activity), and a line with a positive slope can be drawn representing all the values. If benzo[a]pyrene and acetanilide are metabolized principally by the same form of 3-methylcholanthrene-induced cytochrome, one would have observed a cluster of points around the mean activity for each enzyme with a coefficient of variance between 0.10 and 0.25 for either enzyme activity. This was clearly not the case. More than 5-fold differences in aryl hydrocarbon hydroxylase activity and as much as 4-fold differences in acetanilide 4-hydroxylase activity were found among the 55 individual 3-methylcholanthrene-treated HS mice. These fold differences are certainly beyond the magnitude of any experimental error of the enzyme assays. These data are consistent with the possibility that the induction of at least two forms of P-450 by polycyclic aromatic compounds is associated with the murine Ah locus.

Purification of P-450 and P-448 Fractions from 3 Methylcholanthrene-treated B6 Liver—The purified forms of 3-methylcholanthrene-treated B6 hepatic microsomal P-450 was carried out as described under "Experimental Procedures." When the potassium phosphate buffer concentration was increased stepwise (Fig. 2A), two major (and probably several other minor) P-450-rich fractions were eluted. Fractions 17 to 29 and 33 to 40 (designated by the shaded areas) were each combined and concentrated as described in detail under "Experimental Procedures" and are termed for the remainder of this report "P-450" and "P-448," respectively. The specific contents of partially separated P-450 and P-448 were 15.4 and 14.2 nmol of cytochrome/mg of protein, respectively. Electrophoresis of the partially separated fractions (Fig. 2B) showed small amounts of contaminating proteins, at least one heavier and three or four lighter moieties. The estimated molecular weight of both partially separated P-450 and P-448 was between 54,500 and 55,000. This finding differs from previous electrophoretic data (13, 31), indicating that the two major peaks from intact liver microsomes that are inducible by 3-methylcholanthrene or TCDD in the genetically responsive mouse have an estimated mass difference between 300 and 1,000 daltons. It is certainly possible with this sodium dodecyl sulfate-polyacrylamide gel system that a partially solubilized and separated form of P-450 can appear to have a different molecular weight, compared with that same hemoprotein when the intact microsomes are electrophoresed. Contrary to the mouse, however, in rat (13) and rabbit (10) intact liver microsomes, the two major 3-methylcholanthrene-inducible electrophoretic bands have estimated mass differences of about 2,000 and 3,000 daltons, respectively.

Distinctly different patterns between P-450 and P-448 were seen with limited proteolysis of the partially separated cytochromes (Fig. 3). These data indicate that fundamental differences exist in the peptides of these two fractions.

Spectral and Catalytic Properties of P-450 and P-448—The Soret maximum of the dithionite-reduced cytochrome CO complex for the P-448 fraction (Table I) was shifted more than 1.0 nm further to the blue than that for the P-450 fraction. This finding was not unexpected and had been discussed previously for these cytochromes in the intact microsomes of rabbit (10) and rat (13). Aryl hydrocarbon hydroxylase activity was 2-fold greater with the P-450 than the P-448 fraction, and more than 2 times as much NADPH was oxidized by P-450 than P-448 in the presence of benzo[a]-
Characterization of Mouse Liver PI-450 and P-448

Fig. 2. Partial separation of CO-binding cytochrome fractions. A, elution pattern from the hydroxyapatite column as a function of stepwise increases in potassium phosphate buffer concentration. The shaded areas denote those fractions from which PI-450 or P-448 was concentrated. B, electrophoretogram of partially separated PI-450 and P-448 eluted and concentrated from the procedure illustrated in A. Electrophoretic migration is from top to bottom. Each cytochrome (about 5 µg) and a mixture (about 10 µg of each protein) of molecular weight standards (Stds.) were applied to the three wells, respectively, of (1.5 mm x 9.0 cm) analytical gels; from this technique a molecular weight ($M_r$) of approximately 55,000 was estimated for both PI-450 and P-448. Further details are described under “Experimental Procedures.”

Fig. 3. Electrophoretogram of peptides derived from the limited proteolysis of PI-450 (left) and P-448 (right), each at two concentrations of the V8 protease. Electrophoretic migration is from top to bottom. Arrows designate distinct differences between the two partially separated cytochrome fractions. Further details are described under “Experimental Procedures.”

Pyrene as substrate. Acetanilide 4-hydroxylase activity, on the other hand, was 2-fold greater with the P-448 than the PI-450 fraction, and more than 3 times as much NADPH was oxidized by P-448 than PI-450 in the presence of acetanilide as substrate. These data are consistent with previous reports of overlapping substrate specificities of the multiple forms of rabbit or rat P-450 (7, 8, 10–12). An alternative, or additional, explanation would be that we only have attained a partial...
Inhibition of Catalytic Activities by Anti-P-450 and Anti-P-448—Anti-P-450 markedly inhibited aryl hydrocarbon hydroxylase from 3-methylcholanthrene- or TCDD-treated B6 mice (Fig. 4, top), whereas acetanilide 4-hydroxylase activity.

**Table I.** Spectral and catalytic properties of intact microsomes or partially separated P-450 or P-448 from 3-methylcholanthrene-treated B6 liver

<table>
<thead>
<tr>
<th>Characterization of Mouse Liver P-450 and P-448</th>
<th>P-450</th>
<th>P-448</th>
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<tr>
<td>Spectral and catalytic properties of intact microsomes or partially separated P-450 or P-448 from 3-methylcholanthrene-treated B6 liver</td>
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<tr>
<td>Sorbit peak of reduced hemoprotein-CO complex (nm)</td>
<td>448.0</td>
<td>449.3</td>
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<tr>
<td>Specific monooxygenase activity (units/nmol cytochrome)</td>
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<tr>
<td>Aryl hydrocarbon hydroxylase</td>
<td>2170</td>
<td>1650</td>
</tr>
<tr>
<td>Acetanilide 4-hydroxylase</td>
<td>1370</td>
<td>3180</td>
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<tr>
<td>NADPH oxidation in presence of substrate (nmol/nmol cytochrome)</td>
<td>0.47</td>
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<tr>
<td>Benzo[a]pyrene</td>
<td>18.6</td>
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</tr>
<tr>
<td>Acetanilide</td>
<td>0.47</td>
<td>1.5</td>
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<tr>
<td>Substrate difference spectrum</td>
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<td>“Type I”</td>
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<tr>
<td>Benzo[a]pyrene</td>
<td>“Reverse type I”</td>
<td>“Type I”</td>
</tr>
<tr>
<td>Acetanilide</td>
<td>7.7</td>
<td>~0.82</td>
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<tr>
<td>Apparent K, value (μM)</td>
<td>550</td>
<td>&gt;880</td>
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“Values represent determinations on livers pooled from six 3-methylcholanthrene-treated B6 mice.

*Fig. 4.* Effect of anti-P-450 goat serum (left) or anti-P-448 rabbit serum (right) immunoglobulin (IgG) on aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase activities from intact liver microsomes of B6 mice which had previously been treated with 3-methylcholanthrene (MeChol) or TCDD (top) and from intact liver microsomes from untreated control B6 and D2 mice (bottom). Microsomes in each instance had been prepared from livers combined from six animals. 3-Methylcholanthrene or TCDD microsomes (125 μg) or control microsomes (570 μg) were preincubated in a total volume of 450 μl with the designated amounts of anti-P-450 or anti-P-448 immunoglobulin for 10 min at 37°C. The preincubation mixture contained 50 mM Tris-chloride buffer (pH 7.25 for aryl hydrocarbon hydroxylase; pH 7.50 for acetanilide 4-hydroxylase) and 3.3 mM MgCl₂. Following the preincubation, the mixtures were placed on ice, and 450 μl of 50 mM Tris-chloride buffer, containing 0.6 mg of NADPH, 0.6 mg of NADH, 0.7 mg of bovine serum albumin, and 3.3 mM MgCl₂, was added to each preincubation mixture. The mixtures were now ready for the routine monooxygenase assays, as described further under “Experimental Procedures.” For control experiments, designated amounts of preimmune immunoglobulin were incubated instead of the goat or rabbit IgG. The enzyme activity from the control experiments, at each starting activity. The starting aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase activities for control, 3-methylcholanthrene and TCDD-treated B6 mice were 860, 3470, 3270, and 780, respectively, units/mg of microsomal protein. The starting acetanilide 4-hydroxylase activities for control, 3-methylcholanthrene-treated and TCDD-treated B6 mice, and control D2 mice were 860, 3470, 3270, and 780, respectively, units/mg of microsomal protein. Each point represents the average of triplicate determinations that differed from each other by less than 10%. A second experiment in each case yielded similar results. Differences of 20% or more are statistically (p < 0.05) significant.
was minimally affected. Contrariwise, anti-P-448 markedly inhibited acetylcholine 4-hydroxylase from these same animals while not blocking aryl hydrocarbon hydroxylase activity at all. Anti-P-450 blocked TCDD-induced aryl hydrocarbon hydroxylase activity more than 3-methylcholanthrene-induced aryl hydrocarbon hydroxylase activity, whereas anti-P-448 blocked TCDD-induced acetanilide 4-hydroxylase activity less than 3-methylcholanthrene-induced acetanilide 4-hydroxylase activity. These data suggest that TCDD induces more specifically P-450 than P-448.

In control, untreated mice (Fig. 4, bottom), anti-P-450 inhibited both aryl hydrocarbon hydroxylase and acetanilide 4-hydroxylase activities in B6 more so than in D2. These findings suggest that in genetically responsive B6 “controls,” but not in nonresponsive D2 “control” mice, these enzymes are slightly induced by some factor(s) in their environment. This possibility has recently been shown to be the case, in fact, by means of the very sensitive in vitro assay of binding of radioactive benzo[a]pyrene metabolites to deproteinized DNA (34): chemicals apparently in normal laboratory chow induce P-490 in B6 but not in D2, yet between control B6 and D2 differences in aryl hydrocarbon hydroxylase activity or total P-450 content are not detectable.

Anti-P-448 inhibited significant amounts of acetanilide 4-hydroxylase activity in both control B6 and D2 mice while not affecting aryl hydrocarbon hydroxylase in either strain. There are two possible interpretations of these data: (i) P-448 occurs normally in control mouse liver, or (ii) anti-P-448 interacts with one or more forms of “control” P-450.

Inhibition of Catalytic Activities by Other Chemicals—Acetanilide in vitro very effectively inhibited aryl hydrocarbon hydroxylase activity in 3-methylcholanthrene microsomes more so than in control intact microsomes (Fig. 5). No difference in sensitivity to acetanilide was observed for P-450- or P-448-catalyzed aryl hydrocarbon hydroxylase activity.

**Table II**

<table>
<thead>
<tr>
<th>Test compound</th>
<th>Aryl hydrocarbon hydroxylase activity</th>
<th>Acetanilide 4-hydroxylase activity</th>
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<tr>
<td></td>
<td>Intact microsomes*</td>
<td>Reconstituted system</td>
</tr>
<tr>
<td>Acetanilide</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>±</td>
<td>±</td>
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<tr>
<td>Benzo[a]anthracene</td>
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<tr>
<td>α-Naphthoflavone</td>
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<td>+</td>
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* From 3-methylcholanthrene-treated B6 mice, six livers pooled.

Benzo[a]pyrene very effectively blocked acetanilide 4-hydroxylase activity in 3-methylcholanthrene microsomes more so than in control intact microsomes. No important difference in sensitivity to Benzo[a]pyrene was found for P-450- or P-448-catalyzed acetanilide 4-hydroxylase activity.

On the basis of similarities in chemical structure to benzo[a]pyrene, it was postulated that benzo[a]anthracene would be a better inhibitor of aryl hydrocarbon hydroxylase than acetanilide 4-hydroxylase activity. On the same basis, we thought that α-naphthoflavone might be a better inhibitor of acetanilide 4-hydroxylase than aryl hydrocarbon hydroxylase activity. The latter is shown to be the case (Fig. 6, bottom) in both intact microsomes and with the reconstituted assay system. To our surprise, however, (Fig. 6, top), benzo[a]anthracene more effectively blocked 3-methylcholanthrene-induced acetanilide 4-hydroxylase in both intact microsomes and with partially separated P 448 or P 450, compared with the effect of benzo[a]anthracene on aryl hydrocarbon hydroxylase in these experiments.

The relative magnitudes of inhibition by these four chemicals in vitro are summarized in Table II. To date, the most

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**Fig. 5.** Effect of acetanilide (top) on aryl hydrocarbon hydroxylase activity or benzo[a]pyrene (bottom) on acetanilide 4-hydroxylase activity in intact microsomes (left) of control or 3-methylcholanthrene-treated B6 mice and in the reconstituted system (right) containing P-450 or P-448, NADPH-cytochrome P-450 reductase, and dilauroyl-S-phosphotidylcholine. Microsomal protein concentration was 0.5 mg/ml; P-450 or P-448 concentration was 0.1 nmol/ml. The albumin needed for the solubilization of benzo[a]pyrene as substrate (22) was unnecessary for the acetanilide 4-hydroxylase assay.

**Fig. 6.** Effect of benzo[a]anthracene (top) or α-naphthoflavone (bottom) on aryl hydrocarbon hydroxylase or acetanilide 4-hydroxylase activities from intact microsomes (left) of control or 3-methylcholanthrene-treated B6 mice and from the reconstituted system (right). The conditions were the same as those described in the legend to Fig. 5 and are detailed further under “Experimental Procedures.”
Characterization of Mouse Liver PI-450 and P-448

Fig. 7. Analysis of immunoprecipitates by sodium dodecyl sulfate polyacrylamide gel electrophoresis. A, electrophoretogram of intact microsomes from TCDD-treated and control fetal liver and of partially separated PI-450. About 50 μg of microsomal protein and about 2 μg of PI-450 were loaded on the 7.5% gels (1.5 mm x 20 cm). Arrows indicate the protein bands inducible transplacentally by TCDD. From this technique molecular weights (M_r) of approximately 55,000 and 120,000 were estimated for the two major inducible protein subunits. B and C, radioactivity of the gel slices when NaB[3H]-labeled microsomes from TCDD-treated B6 fetuses were immunoprecipitated with anti-PI-450 and anti-P-448, respectively. The immunoprecipitates were dissolved in 50 μl of sodium dodecyl sulfate and boiled briefly with β-mercaptoethanol. Following electrophoresis, 1-mm gel slices were dissolved as previously described (28). Arrows indicate the migration of the heavy chain of goat and rabbit immunoglobulin, respectively. The cytochromes co-migrated with the heavy chain.

effective inhibitor of 3-methylcholanthrene-induced aryl hydrocarbon hydroxylase activity is acetanilide. Furthermore, a-naphthoflavone appears to be a much better inhibitor of P-448-catalyzed aryl hydrocarbon hydroxylase activity than PI-450-catalyzed aryl hydrocarbon hydroxylase activity. This fact has not been appreciated before; whether this finding is also true in rabbit or rat liver should be examined.

Developmental Differences in PI-450 and P-448—During the end of gestation and in the neonatal period of the rat or mouse (13), TCDD-inducible aryl hydrocarbon hydroxylase activity (and its associated PI-450) is expressed 2 to 4 days earlier than TCDD-inducible acetanilide 4-hydroxylase activity (and its associated P-448). These differences in temporal expression between PI-450 and P-448 were studied further with the aid of our newly developed antibodies (Fig. 7). With the 20-cm gels, two major electrophoretic bands are seen to be increased in fetal liver microsomes by TCDD treatment of the pregnant mother (Fig. 7A). The 55,000-dalton band is believed to represent predominantly PI-450 and, to a minor extent, P-448; the function of the 120,000-dalton band is under further investigation. This latter protein is not induced in the adult mouse. Of interest, however, is that an electrophoretic band of approximately the same molecular weight can be seen but was not mentioned in the fetal or newborn rabbit following 3-methylcholanthrene (10) or TCDD (35) treatment.

By means of the in vitro pyridoxal phosphate-NaB[3H] labeling technique (Fig. 7, B and C and Table III), approximately an equal amount of tritium in fetal microsomes and 9 times more tritium in adult microsomes were immunoprecipitated by anti-P-448 than by anti-PI-450. These data may indicate that P-448 is more exposed on the microsomal membrane surface and is, therefore, more readily tritiated during this in vitro method than PI-450. Another possibility is that P-448 contains more lysine and asparagine than PI-450 since this procedure preferentially tritiates the ε amino groups of these amino acids (especially lysine). A third possibility is that a large amount of P-448 is in a catalytically inactive form. More recent experiments involving the in vitro labeling of intact microsomes with [3H]labeled microsomes from TCDD-treated B6 fetuses were immunoprecipitated with anti-PI-450 and anti-P-448, respectively. The immunoprecipitates were dissolved in 50 μl of sodium dodecyl sulfate and boiled briefly with β-mercaptoethanol. Following electrophoresis, 1-mm gel slices were dissolved as previously described (28). Arrows indicate the migration of the heavy chain of goat and rabbit immunoglobulin, respectively. The cytochromes co-migrated with the heavy chain.

4 M. Negishi and D. W. Nebert, unpublished data.
Table III
Comparison of relative amounts of Pi-450 and P-448 in B6 mouse liver microsomes from TCDD-treated fetuses and 3-methylcholanthrene-treated adults

<table>
<thead>
<tr>
<th></th>
<th>Fetus</th>
<th>Adult</th>
<th>Fetus/Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific aryl hydrocarbon hydroxylase activity (units/mg microsomal protein)</td>
<td>1,250</td>
<td>2,060</td>
<td>0.61</td>
</tr>
<tr>
<td>Specific acetanilide 4-hydroxylase activity (units/mg microsomal protein)</td>
<td>390</td>
<td>3,500</td>
<td>0.11</td>
</tr>
<tr>
<td>Anti-P-450 immunoprecipitate (cpm)</td>
<td>1,440³</td>
<td>2,110</td>
<td>0.68</td>
</tr>
<tr>
<td>Anti-P-448 immunoprecipitate (cpm)</td>
<td>1,889³</td>
<td>18,550</td>
<td>0.08</td>
</tr>
</tbody>
</table>

³ Radioactivity of total trichloroacetic acid-precipitable material was 2.2 x 10³ and 2.8 x 10⁶ cpm/mg of microsomal protein for fetal and adult microsomes, respectively. Our observed values of 1130 and 1180 cpm for fetal anti-P-450 and anti-P-448 immunoprecipitates, respectively, are, therefore, adjusted to the adult total microsomal radioactivity so that a valid fetus/adult ratio could be made.

Variations of more than 5-fold for 3-methylcholanthrene-induced aryl hydrocarbon hydroxylase activity and as much as 4-fold for 3-methylcholanthrene-induced acetanilide 4-hydroxylase activity among 55 individual HS mice (Fig. 1) most likely indicate that the structural genes, and/or the minor regulatory genes, for P-450 and P-448 are not linked. It must be kept in mind that, although the cytosolic receptor is the major regulatory gene product (reviewed in Ref. 3), a minimum of six alleles and at least two loci are needed (39) to explain the inducibility of aryl hydrocarbon hydroxylase by 3-methylcholanthrene in appropriate genetic crosses among 12 inbred mouse strains. It is, therefore, quite likely that, during the development of these HS mice, new phenotypes have resulted from recombination of these various minor regulatory alleles, dependent upon dominance, epistasy, etc. A similar example would be Bateson’s famous case (40) of crossing two white varieties of sweet pea and obtaining a purple F₁ and a 9:7 ratio of colored:white among the F₂ population. An alternative, or additional, possibility is that a variant in aryl hydrocarbon hydroxylase (and/or acetanilide 4-hydroxylase) induction arose by mutation sometime during the formation or maintenance of the HS stock and has persisted until the present, without these stock becoming homozygous for either the usual allele or the new one. Given the outbreeding system used, the HS mice could remain heterogeneous for many years. It is interesting that aryl hydrocarbon hydroxylase specific activities (between 150 and 1200) in 3-methylcholanthrene-treated HS mice are about 2 or more times less than those of 3-methylcholanthrene-treated B6 mice (usually between 1800 and 3000) and that acetanilide 4-hydroxylase specific activities (between 300 and 1200) in 3-methylcholanthrene-treated HS mice are more than 2 times less than those of 3-methylcholanthrene-treated B6 mice (usually between

Discussion
The data herein provide direct evidence in support of previous indirect findings from this laboratory, i.e. that P-448 is not the cytochrome most closely associated with 3-methylcholanthrene-inducible aryl hydrocarbon hydroxylase activity. In other words, the polycyclic hydrocarbon-inducible form of P-450 having the greatest amount of high spin P-450 iron (36) and the largest hypochromic shift in the Soret peak of the reduced hemoprotein-CO complex is not the monoxygenase responsible for the most efficient metabolism of the chemical carcinogen benz(a)pyrene.

It should be emphasized that the P-450 research is plagued with the problems of incomplete separation of one form of cytochrome from another. These problems are reminiscent of early studies of immunoglobulin synthesis (37). The discovery that large amounts of a single soluble antibody are secreted from each monoclonal marine myeloma or plasmacytoma has led to much more success in studying the immune response than the P-450 induction response. Still, overlapping antigen specificities occur among many different antibodies (38), very similar to the overlapping substrate specificities among the different forms of P-450. In view of the methods by which the eukaryote can respond to the diversity of approximately 10⁶ antigens, it should be kept in mind that there similarly may exist as many forms of P-450 as there are P-450 inducers of varying chemical structures (37). When an antigen stimulates the synthesis of its specific antibody, dozens of other less specific antibodies can be concomitantly stimulated (38). The same phenomenon may occur in the case of P-450 induction. 3-methylcholanthrene stimulates most specifically the synthesis of P-450, but other forms of P-450 such as P-448 are (accidentally?) increased at the same time. The data in this report simply demonstrate that, when two major fractions of 3-methylcholanthrene-inducible P-450 are partially separated from one another and then compared, differences in genetic, peptide sequence, spectral, catalytic, immunologic, inhibitor-specific, and developmental parameters can all be demonstrated.
2500 and 3500). During outbreeding or random breeding, this “tendency” for aryl hydrocarbon hydroxylase induction to be less than that in highly inducible inbred strains, and to become less as a function of time, has been recently reported (41) and suggests suppressor properties of one or more of the minor regulatory genes expressed during heterozygosity.

We found differences between intact microsomes and partially separated P, 450 or P, 448 with respect to the acetylneidic difference spectrum (Table I) and the inhibition of aryl hydrocarbon hydroxylase activity by naphthoflavone (Fig. 6 and Table II). It is perhaps not surprising to find that detergent-solubilized highly purified cytochrome P, 450 and P, 448 gave no detectable difference in molecular weight (Fig. 2B). Perhaps the nature of at least one of these cytochromes has changed during its purification. Proteolysis or loss of a carbohydrate moiety, for example, might occur during detergent treatment plus column chromatography. Alternatively, the P, 450 and P, 448 described in this report may not actually represent the two major forms of 3-methylcholanthrene-induced hemoprotein observed by electrophoresis of the intact microsomes (31).

A further consideration is for us to develop a better understanding of which benz[a]pyrene metabolic pathway is most inducible by polycyclic aromatic compounds. “Aryl hydrocarbon hydroxylase activity” in this report is principally the protein observed by electrophoresis of the intact microsomes (31).

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