Identification of the Major Cytochrome P-450 Form Transplacentally Induced in Neonatal Rabbits by 2,3,7,8-Tetrachlorodibenzo-p-dioxin*

Richard L. Norman, Eric F. Johnson, and Ursula Muller-Eberhard

From the Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037

Transplacental induction of cytochrome P-450 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was studied in newborn rabbit liver. This treatment increased a single cytochrome species in microsomes of newborn rabbit liver as demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This cytochrome species had the same mobility as cytochrome P-450 form 6, isolated from liver microsomes of TCDD-treated adult rabbits. The identity of the major component was confirmed by a comparison of its peptide fingerprint with that of purified adult form 6. This assignment was further supported by immunological studies which showed that monospecific antibody developed against adult form 6 reacted with microsomes from TCDD-treated newborns but not with the microsomes from control newborns. Benzo[a]pyrene hydroxylase and 7-ethoxycoumarin O-deethylase activities are associated with purified form 6 reconstituted with purified reductase and phospholipid. TCDD treatment increased these enzyme activities in newborn microsomes. Furthermore, these enzyme activities were strongly inhibited by α-naphthoflavone both in the reconstituted system and in microsomes from TCDD-treated newborns. These results demonstrate that microsomal metabolism in TCDD-treated newborn rabbits reflects the catalytic properties of the predominant cytochrome P-450 species, which is form 6.

In the adult rabbit, two forms of cytochrome P-450 are induced by TCDD, form 4 which is the major species induced by TCDD and form 6. This difference in the response of adult and neonatal rabbits to TCDD suggests that an age-dependent factor(s) controls the induction of form 4. Variations in cytochrome composition with development and in response to inducers will affect xenobiotic metabolism and may have important consequences on the susceptibility of an animal to chemical carcinogenesis.

Cytochrome P-450 is the terminal oxidase in an enzyme system which metabolizes a wide variety of drugs, carcinogens, and environmental chemicals (1, 2). Recent work has established the existence of multiple forms of cytochrome P-450 in various species (3-15). This laboratory has reported the resolution of several forms of cytochrome P-450 from liver microsomes of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-treated adult rabbits. The major cytochrome P-450 species, form 4, and a second species, form 6, were highly purified from this source (3-6). A variety of criteria has been established in this laboratory by which these two forms can be identified and distinguished from other known forms. Forms 4 and 6 are immunologically distinct, have different molecular weights on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and have different peptide "fingerprints" after limited proteolysis. Although certain functional properties of the two forms are similar, such as a sensitivity to inhibition by α-naphthoflavone, there are definite differences in the substrate specificity of each form. Form 6 preferentially metabolizes benzo[a]pyrene while form 4 is more active than the other forms in the turnover of acetanilide.

Variation in the metabolism of steroids, drugs, and carcinogens with species, tissue, and exposure to inducers probably reflects variations in the relative proportions of functionally different forms of cytochrome P-450. We are interested in the relationship between variations in metabolism and cytochrome P-450 composition during the course of development. In the rabbit, cytochrome P-450 and associated enzymatic activities are not detectable in fetal liver until the last third of gestation (16-18). These enzyme activities are present at low levels at birth (16-19) and rapidly increase to adult levels during the neonatal period (18-21). Variations in the time of appearance of the enzyme activities associated with cytochrome P-450 suggest that the individual forms of the cytochrome are genetically expressed at different stages of development (18, 22, 23). Efforts have been made to alter the cytochrome P-450 composition in fetal and neonatal rabbits through exposure to inducers (16, 17, 19, 24). In the rabbit, phenobarbital appeared to induce the same enzyme activities during the perinatal period that it induced in the adult (16).

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1 The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; SDS, sodium dodecyl sulfate.

2 In conformance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature which suggests that a numerical system based on gel electrophoresis be used to designate multiple enzyme forms, the following system of nomenclature for multiple forms of cytochrome P-450 in the rabbit was employed in this paper. The major phenobarbital-induced species (M. = 48,500) is designated form 2, the major TCDD-induced or β-naphthoflavone-induced species (M. = 54,500) is designated form 4, and the minor TCDD-induced species (M. = 57,000) is designated form 6. This nomenclature corresponds to that used by Coon and co-workers (7, 8) and to that of Guengerich (10) except that the designation Lm (liver microsomal) has been omitted. Forms a, b, and c described by Johnson and Muller-Eberhard (3-6) correspond to forms 1, 6, and 4, respectively, in this system.

3 E. F. Johnson, M. C. Zounes, and U. Muller-Eberhard, manuscript in preparation.

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17, 19), but Atlas et al. (24) have recently observed that the polycyclic aromatic hydrocarbon inducer, 3-methylcholanthrene has differential effects on adult and neonatal rabbit cytochrome P-450 monoxygenase activities. These results indicated that polycyclic aromatic hydrocarbons might induce different cytochrome P-450 species at different stages of development.

We report here on studies of the effects of TCDD, a very potent inducer of the polycyclic aromatic hydrocarbon class (25), on cytochrome P-450 composition in adult and newborn rabbit liver microsomes. TCDD injected in the dam transplacentally induced a single form of cytochrome P-450 in liver microsomes isolated from the newborn offspring. By three independent criteria, this form was identical with form 6 isolated from the liver microsomes of TCDD-treated adult rabbits. In contrast, form 4 was the major species induced in TCDD-treated adults. This difference suggests that an age-dependent factor(s) controls the induction of form 4 in the rabbit.

EXPERIMENTAL PROCEDURES

Materials—TCDD was a gift from the Dow Chemical Co, Dr. Harry Gelboin of the National Cancer Institute kindly provided the 3-hydroxybenzo[a]pyrene. All materials for SDS-polyacrylamide gels were purchased from Bio-Rad Laboratories except for Coomassie brilliant blue R and bromophenol blue which were purchased from Sigma Chemical Co. and Eastman Kodak Co., respectively. Other materials used in these experiments were obtained from the following suppliers: Aldrich Chemical Co., benzo[a]pyrene (gold label), acetanilide (gold label), p-hydroxyacetanilide, 3,3',5,5'-tetramethylbenzidine (gold label), and n nepthoflavone; J. T. Baker Chemical Co., 1,4-dioxane; Burdick and Jackson Laboratories Inc., hexane, methanol, and acetone (all distilled in glass); Matheson, Coleman and Bell, diethyl sulfoxide; Miles Laboratories Inc., Staphylococcus aureus Vx protease; Pierce Chemical Co., 7-ethoxyresorufin and resorufin; Sigma Chemical Co., carbonic anhydrase, egg albumin (grade V), bovine serum albumin (fatty acid-free), phosphorylase a, papain, sodium cholate, sodium deoxycholate, and quinine sulfate.

Animals—A breeding colony of female New Zealand White rabbits weighing 3 to 4 kg was established. On the 24th day after successful mating, the female was injected subcutaneously with 30 nmol/kg of TCDD (0.3 mg/kg) dissolved in dioxane. Male rabbits weighing 3 to 5 kg injected subcutaneously with 30 nmol/kg of TCDD (0.3 mg/kg) were used to prepare adult liver microsomes. Control animals were either untreated or injected subcutaneously with an equivalent volume of the dioxane vehicle (0.1 ml/kg). The animals were housed individually over Ab-sorb-dri bedding and allowed free access to food and water. Animals used to prepare adult liver microsomes were fasted for 24 h prior to killing.

Microsomes—Male rabbits were killed on the 5th day after the injection of TCDD or dioxane, and liver microsomes were prepared as described by van der Hoeven and Coon (26). Newborn rabbits were killed within 12 h of birth (birth usually occurred on Day 31 or 32 of the gestation period). Livers of animals of the same sex were pooled and microsomes were prepared as described above, except that the livers were homogenized in a Potter-Elvehjem Teflon-glass homogenizer and the initial centrifugation was at 8000 x g for 30 min. The final microsomal pellet was resuspended by hand with a Potter-Elvehjem Teflon-glass homogenizer in 20% glycerol, 0.1 mM EDTA, 10 mM Tris/acetate, pH 7.4.

Since no significant sex-related differences in SDS-polyacrylamide gel patterns or enzyme activities were observed in either control or TCDD-treated rabbits, data from both sexes were combined. Untreated controls and dioxane-treated controls also had similar SDS-polyacrylamide gel patterns and similar levels of enzyme activity. Consequently, results from both untreated and dioxane-treated rabbits were grouped together to obtain control values.

Resolution of Cytochrome P-450 Forms—Forms 1, 4, and 6 were isolated from liver microsomes from TCDD-treated adults as described by Johnson and Muller-Eberhard (3-5). Form 2 was isolated according to the procedure of van der Hoeven and Coon (26) with some modifications from liver microsomes of rabbits given 0.1% phenobarbital (sodium salt) for 6 days in their drinking water.

Cytochrome P-450 Determination—The cytochrome P-450 content was estimated from the CO dithionite-reduced cytochrome P-450 difference spectrum determined with a Cary 118 C double beam spectrophotometer according to the procedure of Omura and Sato (27). An extinction coefficient of 91 mM cm⁻¹ was used in the calculation of the concentration of cytochrome P-450.

Protein Assay—The protein content was estimated by the procedure of Lowery et al. (28) following precipitation of the sodium deoxycholate (500 µl of 0.013% solution) with trichloroacetic acid (500 µl of 10%) to reduce interference from buffer components (29). Bovine serum albumin was used as a standard.

Polycyramide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (30) except that 0.1 mM dithiothreitol was substituted for mercaptoethanol. Twenty to thirty micrograms of microsomal protein were applied to each well of a 3-mm thick slab gel. After electrophoresis, the slab gel was stained for protein with Coomassie blue as described by Haugen and Coon (8). A mixture of molecular weight standards, carbonic anhydrase (29,000), egg albumin (43,000), bovine serum albumin (68,000), and phosphorylase a (94,000), was electrophoresed in a separate well. For densitometry measurements, sample tracks were cut out of the destained slab gel and scanned at 580 nm on a Beckman DU spectrophotometer equipped with a Gilford gel scanning apparatus. Polycyramide gels were also prepared and stained for heme-associated peroxidase as described by Thomas et al. (31).

Peptide Mapping—The basic procedure was that of Cleveland et al. (32) with slight modifications. Microsomal components were separated on SDS-polyacrylamide gels as described above, stained, washed, and the component of interest excised from the gel. After soaking the gel slices 30 min in 0.1% SDS, 10% glycerol, 0.125 M Tris/chloride, pH 8.8, the slices were placed on top of a 2% acrylamide disc gel sealed at the bottom with dialysis tubing. The protein was electrophoresed through the 5% acrylamide gel and into the dialysis bag. The sample was concentrated by precipitating an aliquot of the contents of the dialysis bag with trichloroacetic acid (10% final concentration), and the precipitate was dissolved in 0.1% SDS, 10% glycerol, 0.001% bromophenol blue, and 0.1% mercaptoethanol. The sample was then electrophoresed at a final concentration of 0.33 mg/ml. The dissolved sample was incubated for 30 min at 37°C with S. aureus Vx protease or papain, final concentration 60 or 1 µg/ml, respectively. Dithiothreitol was then added to a final concentration of 0.1 M and the samples were heated 2 min at 100°C. Ten micrograms of sample protein were loaded into individual wells in a 12.5% acrylamide slab gel, electrophoresed, and stained as described previously.

Immunological Techniques—Antibody directed against form 6 was produced, as described by Johnson and Muller-Eberhard (3) for the production of anti-form 4, by immunizing goats with form 6 resolved as described by Johnson and Muller-Eberhard (3) for the production of anti-form 4. Anti-form 6 was produced, as described by Johnson and Muller-Eberhard (3) for the production of anti-form 4, by immunizing goats with form 6 resolved as described by Johnson and Muller-Eberhard (3) for the production of anti-form 4.

Enzyme Activity—Benzo[a]pyrene hydroxidase was assayed in 3 mM MgCl₂, 80 µM benzo[a]pyrene (2.0 mM in methanol), 50 mM Tris/chloride, pH 7.4. The reaction was initiated by the addition of 100 µl of 20 mM NADPH. The final reaction volume was 1.0 ml. After a 20-min incubation at 37°C, the phenolic metabolites were extracted with chloroform, and the radioactivity was measured by the method of Davis and Vetel [68]. Benzo[a]pyrene hydroxidase was determined using quinine sulfate in 0.1 M H₂SO₄ as a standard. The fluorescence emission of an authentic sample of 3-hydroxybenzo[a]pyrene relative to quinine sulfate was used in the determination of the amount of 3-hydroxybenzo[a]pyrene formed. 7-Ethoxyresorufin O-deethylase was assayed in 50 mM potassium phosphate buffer, pH 7.7, by the procedure of Burke and Mayer [24]. 7-Ethoxyresorufin (25 µM in dimethyl sulfoxide) was added to give a final substrate concentration of 15 µM in a final reaction volume of 2.0 ml. The reaction was initiated with 10 µl of 50 µM
TCDD induces cytochrome P-450 in both adult and newborn rabbit liver microsomes. In adult rabbits, as shown in Table I, TCDD treatment resulted in a 2-fold increase in cytochrome P-450 content over that of control adults. TCDD injected into the dam transplacentally increased the cytochrome P-450 content of newborn rabbit liver microsomes 5-fold, from 0.3 nmol/mg of protein to 1.6 nmol/mg of protein. These levels of cytochrome P-450 are similar to those found in adult control animals.

TCDD treatment also affected the absorbance maximum of the CO dithionite-reduced cytochrome P-450 complex in the Soret region. The absorbance maximum of this complex in microsomes from control newborns was at 452 nm, while that of control adults was at 450 nm. Microsomes from TCDD-treated adult and newborn rabbits both exhibited an absorbance maximum at 448 nm.

Several forms of cytochrome P-450 have been purified from adult rabbit liver (3-12) including forms 4 and 6 isolated in this laboratory from TCDD-treated animals (3-5). These forms have different molecular weights and SDS-polyacrylamide gel electrophoresis, which separates individual components of a complex mixture on the basis of molecular weight, was employed to evaluate the effect of TCDD treatment on the cytochrome P-450 composition of newborn microsomes. An SDS-polyacrylamide gel pattern typical of microsomes from control and TCDD-treated newborns is illustrated in Fig. 1. TCDD appeared to induce a single component in newborn microsomes. This point was clearly demonstrated when absorbance profiles of microsomes from control and TCDD-treated newborns were compared in Fig. 2a; TCDD treatment significantly increased one component. A direct comparison in Fig. 1 of the mobility of the principal component in microsomes from TCDD-treated newborns with those of purified adult forms 4 and 6 showed that this component had the same mobility as form 6 (apparent M, = 57,000).

Densitometric scans of microsomes from control and TCDD-treated adult rabbits shown in Fig. 2b, revealed that TCDD induced two forms in adult rabbit liver microsomes, forms 4 and 6. Microsomes from newborn rabbits contained a species with a mobility similar to that of adult form 4. However, in contrast to adult rabbits, TCDD treatment did not appear to induce this species in newborn microsomes.

We attempted to identify hemeproteins in SDS-polyacrylamide slab gels prepared and stained for heme-associated peroxidase activity according to Thomas et al. (31). Several components in microsomes from TCDD-treated newborns including the major TCDD-induced species stained for heme. In a control experiment, purified form 4 and several non-TCDD-induced hemeproteins, the molecular weight standards, were electrophoresed together. Form 4 and the two standards with lower molecular weights than form 4 stained for heme. Apparently, heme was lost from the cytochrome during the procedure and was bound nonspecifically to the other proteins, complicating the interpretation of results obtained with this technique.

To further establish the identity of the major cytochrome P-450 species in microsomes from TCDD-treated newborns, peptide fingerprints of this component were compared to those of purified adult forms of cytochrome P-450. The principal component in microsomes from TCDD-treated newborns was separated from other components in the crude preparation by SDS-polyacrylamide gel electrophoresis and then incubated with either S aureus V8 protease or papain. The molecular weight distribution of the peptides from the protease-digested neonatal component was compared on SDS-polyacrylamide gels to that of peptides from protease-digested purified adult forms 1, 2, 4, and 6. Fig. 3 illustrates two important points. First, the major peptides (arrowheads) from the protease-treated, TCDD-induced, neonatal component corresponded closely to those of adult form 6 but not to the major peptides of adult forms 1, 2, and 4. This close correspondence in peptide pattern demonstrated that TCDD

NADPH. The rate of formation of the product, resorufin, was followed fluorimetrically at 30°C (excitation, 574 nm; emission, 586 nm). The fluorescence of a known amount of resorufin added at the end of each assay was used to determine the rate of product formation. Acetanilide 4-hydroxylase was assayed by the method of Krich and Staunton (35) with p-hydroxyacetanilide as the standard. The effect of the inhibitor a-naphthoflavone on the above reactions was determined by adding 10 μl of the appropriate concentration of a-naphthoflavone in methanol to the assay mixture and comparing that activity to a sample containing 10 μl of methanol. Except for the turnover of biphenyl, the values in Fig. 5 were taken from previous publications (3, 5, 6) which describe the reconstitution system and the assay procedures. A manuscript describing the details of the biphenyl hydroxylase assay procedure is in preparation.

RESULTS

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TABLE I

<table>
<thead>
<tr>
<th>Source of microsomes</th>
<th>Specific activity*</th>
<th>CO reduced cytochrome</th>
<th>Absorbance maximum</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg protein</td>
<td>mm</td>
<td>nm</td>
</tr>
<tr>
<td>Newborn control</td>
<td>0.3 ± 0.1</td>
<td>452</td>
<td></td>
</tr>
<tr>
<td>Adult control</td>
<td>1.8 ± 0.2</td>
<td>450</td>
<td></td>
</tr>
<tr>
<td>Newborn TCDD-treated</td>
<td>1.5 ± 0.2</td>
<td>448</td>
<td></td>
</tr>
<tr>
<td>Adult TCDD-treated</td>
<td>3.7 ± 0.6</td>
<td>448</td>
<td></td>
</tr>
</tbody>
</table>

* The mean and standard deviation from a total of six preparations from three newborn litters or from at least five preparations of adult microsomes.

R. L. Norman and E. F. Johnson, unpublished observations.
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treatment preferentially induced form 6 in newborn rabbits. Second, the peptide fingerprint of each purified form was unique, establishing the usefulness of this technique for the identification of individual cytochrome P-450 forms in crude preparations.

Microsomes from newborn and adult rabbits were also characterized immunologically. Antibody directed against form 6 was first tested against purified preparations of forms 1, 2, 4, and 6. The antibody reacted only with form 6 (Fig. 4a), indicating that it recognized a unique, form 6-specific antigen. Microsomes from adult and newborn rabbits were then tested with this antibody to determine whether TCDD treatment resulted in the expression of form 6. In experiments not shown, microsomes from newborn controls did not react with antibody directed against form 6, while microsomes from adult controls reacted weakly. TCDD caused a significant increase in form 6, as evidenced by the line of complete identity observed in Fig. 4b between purified adult form 6 and a component of both TCDD-treated adult and newborn microsomes. Monospecific antibody against form 4 has been shown previously to react with microsomes from both control and TCDD-treated adults (3). This antibody failed to react with microsomes from either control newborns or, as illustrated in Fig. 4b, with microsomes from TCDD-treated newborns. The double diffusion plates were stained for heme-associated peroxidase activity prior to staining for protein. All precipitin lines stained for heme which indicated that the antigens in TCDD-treated microsomes precipitated by either anti-form 4 or anti-form 6 were hemeproteins. In double diffusion plates stained with Coomassie blue, a second faint line of identity was observed between microsomes from TCDD-treated newborns and adult form 6. This second line was not visible in plates stained for heme-associated peroxidase activity. These findings could indicate that anti-form 6 is not monospecific or that it was reacting with apocytochrome P-450 form 6. However, the failure to observe a reaction with the peroxidase stain may mean only that this stain is not sensitive enough to detect the second line. The cause of this second line is currently under investigation.

Having established the identity of the principal form of cytochrome P-450 in microsomes from TCDD-treated new-
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TCDD treatment also increased acetanilide 4-hydroxylase activity in microsomes from newborn rabbits. This activity is associated with form 4 (Fig. 6). Consequently, the increased metabolism of acetanilide could be due to the induction of form 4. However, form 4 was not detected immunologically in microsomes from TCDD-treated newborns, nor did SDS-polyacrylamide gel electrophoresis demonstrate an increase in a component with the same mobility as form 4. Therefore, it is probable that the increase in acetanilide turnover observed after TCDD treatment is due to the large increase in form 6 which metabolizes acetanilide at a lower but still appreciable rate.

In contrast to the inhibition of benzo[a]pyrene metabolism by α-naphthoflavone in microsomes from TCDD-treated newborn rabbits, α-naphthoflavone stimulated benzo[a]pyrene turnover in control newborn microsomes as illustrated in Fig. 7. The inhibitory effect of α-naphthoflavone on these two activities in microsomes from control newborns (light density bars) and newborn (light density bars) rabbits. Activities from TCDD-treated (T) and control (C) rabbits were compared. The assay procedures were described under “Experimental Procedures.” The values are the averages of three or more determinations.

Fig. 6. Induction of liver microsomal cytochrome P-450 monooxygenase activities in adult (heavy density bars) and newborn (light density bars) rabbits. Activities from TCDD-treated (T) and control (C) rabbits were compared. The assay procedures were described under "Experimental Procedures." The values are the averages of three or more determinations.
The greatest effect was observed at 10^{-4} \text{ M } \alpha\text{-naphthoflavone, the highest concentration of } \alpha\text{-naphthoflavone tested. This concentration of } \alpha\text{-naphthoflavone was an order of magnitude greater than that which produced maximal inhibition of benzo[a]pyrene metabolism in microsomes from TCDD-treated newborns. In fact, the stimulation by } \alpha\text{-naphthoflavone may explain the unexpected increase in benzo[a]pyrene hydroxylase activity observed in microsomes from TCDD-treated newborns at } 10^{-4} \text{ M } \alpha\text{-naphthoflavone (Fig. 7b). The mechanism behind the stimulatory effect of } \alpha\text{-naphthoflavone is not known but it has been previously observed in microsomes from control adult rabbits, }^{7} \text{ rats (36), and humans (37). The stimulation of benzo[a]pyrene hydroxylase activity in control microsomes by } \alpha\text{-naphthoflavone suggests the presence of additional forms of cytochrome P-450 in rabbit liver which participate in the metabolism of benzo[a]pyrene.}

### DISCUSSION

This study of the effects of TCDD on neonatal rabbit liver cytochrome P-450 has provided information on two important aspects of the cytochrome P-450 monooxygenase system, the induction of individual cytochrome forms and the relationship between the functional properties of multiple forms and microsomal metabolism. To establish the induction of a particular cytochrome P-450 form, it is first necessary to identify the specific form induced. We have isolated three forms of cytochrome P-450, forms 2, 4, and 6, from adult rabbit liver (3-6) and have established several criteria such as molecular weight and peptide fingerprint by which these forms can be identified. These criteria were employed to evaluate the effect of TCDD on microsomes from adult and newborn rabbit liver. SDS-polyacrylamide gel electrophoresis resolves individual components on the basis of their molecular weights and can be applied to crude preparations as well as to purified proteins. Components with the same mobilities on SDS-polyacrylamide gels as those of the purified forms 4 and 6 were present in microsomes isolated from TCDD-treated newborn rabbit liver. Only one component, that with the same mobility as form 6, appeared to be induced. This observation was supported by densitometry measurements which demonstrated a substantial increase in a single protein component after TCDD treatment. The identity of this component was confirmed by a comparison of its peptide fingerprint with that of form 6. This assignment was supported by immunological studies; antibody directed against form 6 reacted with neonatal microsomes only after the animals had been treated with TCDD. Thus, by these three criteria, the single neonatal cytochrome P-450 form induced by TCDD is identical with adult form 6.

Form 4 is the major cytochrome P-450 species induced by TCDD in adult rabbit liver microsomes. Although there was a component with the same mobility as form 4 present in newborn microsomes, no increase in this species was detected by SDS-polyacrylamide gel electrophoresis after exposure to TCDD. Furthermore, antibody directed against adult form 4 did not react with microsomes from control or TCDD-treated neonates suggesting that either the amount of form 4 is too low to be detected by this method or that the neonatal component is different from adult form 4. The results of both SDS-polyacrylamide gel electrophoresis and immunological studies indicated that TCDD did not induce form 4 in neonatal rabbits.

In contrast to the neonate, TCDD induces both forms 4 and 6 in adult rabbit liver. Densitometric scans of SDS-polyacrylamide gels of microsomes from TCDD-treated adults demonstrated a substantial induction of forms 4 and 6. These scans also showed that the amount of form 4 in microsomes from TCDD-treated adults was greater than that of form 6. Furthermore, antibodies directed against form 4 and against form 6 reacted strongly with microsomes from TCDD-treated adults, indicating the presence of forms 4 and 6 after TCDD treatment.

Comparison of the effects of TCDD on the expression of forms 4 and 6 in the rabbit has established that form 4 is the major TCDD-induced species in adult microsomes while form 6 is the single TCDD-induced cytochrome form in newborns. This difference suggests that the induction of form 4 in rabbit liver is controlled by an age-dependent factor(s) which allows expression of form 4 in response to an inducer only after a particular developmental stage has been reached. It should be noted that the term induction has been used in this paper to describe the effect of TCDD on rabbit liver cytochrome P-450, but the mechanism responsible for the increase in a specific cytochrome is unknown. There is evidence in other species that TCDD or other inducers of the polycyclic aromatic hydrocarbon class cause increased synthesis of cytochrome P-450 (38, 39). However, post-translational processing or decreased catabolism are other possible explanations for the increase in a particular form of cytochrome P-450.

If the kinetic properties of microsomes reflect those of its constituent cytochromes, the induction of a particular cytochrome form(s) should affect microsomal metabolism. This appears to be the case for microsomes from TCDD-treated newborns. A single species of cytochrome P-450, form 6, is induced to a predominant level and the changes in the substrate specificity and inhibitor sensitivity of newborn microsomes reflect this dominance. For example, benzo[a]pyrene hydroxylase and 7-ethoxyresorufin O-deethylase activities, preferentially catalyzed by form 6 in a reconstituted system, were increased 10-fold or more in newborns by TCDD treatment. Furthermore, both of these activities in microsomes from TCDD-treated newborns were strongly inhibited by \( \alpha \text{-naphthoflavone} \). These results also demonstrate that the properties of an individual form observed in a reconstituted system are intrinsic to that form and not artifacts of the isolation or reconstitution procedures.

Atlas et al. (24) have recently reported the induction of several monooxygenase activities in neonatal rabbits by 3-methylcholanthrene. They observed a substantial increase in benzo[a]pyrene hydroxylase activity and a smaller increase in biphenyl 4-hydroxylase activity. In experiments not reported here, we have observed a similar increase in biphenyl 4-hydroxylase activity in microsomes from TCDD-treated newborns. Atlas et al. (24) also detected biphenyl 2-hydroxylase activity, but only in the late term fetus and in the neonate up to 2 weeks of age. SDS-polyacrylamide gel electrophoresis of microsomes from 3-methylcholanthrene-treated neonates revealed the induction of a 57,000 molecular weight component. They did not identify this component, but the increased benzo[a]pyrene hydroxylase and biphenyl 4-hydroxylase activities are consistent with our results and suggest that the 3-methylcholanthrene-induced component is form 6. We have not been able to detect biphenyl 2-hydroxylase activity in microsomes from TCDD-treated neonates. This could be due to differential effects of the two inducers on either the cytochrome P-450 composition or on the enzymes which conjugate the products of the cytochrome P-450 monooxygenase system. For example, TCDD may be a more effective inducer than 3-methylcholanthrene of the transferases which conjugate hydroxylated metabolites. The induction of a transferase with a preference for 2-hydroxybiphenyl could explain why we are unable to detect this metabolite in heptane extracts of the reaction mixture. Experiments are in progress to answer this point.
In other cases, especially where a single cytochrome species does not predominate, the relationship between cytochrome composition and microsomal metabolism can be obscured by several factors including the overlapping substrate specificity of multiple forms and secondary metabolism of the cytochrome P-450 monooxygenase products. Benzo[a]pyrene metabolism illustrates some of the difficulties encountered in trying to correlate cytochrome composition with metabolism. We have established by SDS-polyacrylamide gel electrophoresis and immunological studies that form 6 is induced in adult rabbit liver by TCDD. Since form 6 preferentially metabolizes benzo[a]pyrene, induction of form 6 should result in increased microsomal metabolism of benzo[a]pyrene. Instead, TCDD treatment only slightly increased the specific activity of benzo[a]pyrene hydroxylase in adult liver microsomes. However, it did alter the effect of α-naphthoflavone on microsomal benzo[a]pyrene hydroxylase activity. In control adult microsomes, α-naphthoflavone stimulated benzo[a]pyrene hydroxylase; after TCDD treatment, α-naphthoflavone partially inhibited this activity. This reversal in the effect of α-naphthoflavone is consistent with the induction by TCDD of an α-naphthoflavone-sensitive form, such as form 6. The presence in rabbit liver microsomes of an additional form(s), as yet uncharacterized, which shows overlapping substrate specificity with form 6 has been suggested by studies with α-naphthoflavone. Possibly, a reduction in the amount of the uncharacterized form(s) occurs simultaneously with the induction of form 6 by TCDD. Such a change in cytochrome P-450 composition could result in a reversal in the effect of α-naphthoflavone on benzo[a]pyrene metabolism without altering the turnover rate.

Secondary metabolism, for example, the conversion of epoxides to diols, and the conjugation of hydroxylated products with glutathione, sulfate, and glucuronic acid, may also affect the relationship between microsomal metabolism and cytochrome P-450 composition. The levels of the enzymes which catalyze these reactions can vary with development (40) and are influenced by exposure to inducers (41). The formation of conjugates of several products of benzo[a]pyrene metabolism, including 3-hydroxybenzo[a]pyrene has been observed (42–44). Since benzo[a]pyrene hydroxylase activity is usually assayed by measuring the production of hydroxylated metabolites, primarily 3-hydroxybenzo[a]pyrene, conjugation of these metabolites would reduce the rate of benzo[a]pyrene turnover and further complicate studies on benzo[a]pyrene metabolism.

It should also be recognized that the relationship between the turnover rate in microsomes for a particular substrate and the concentration of a specific cytochrome form in those microsomes is unknown. In TCDD-treated neonates, form 6 has been induced to such a large extent and the background levels of the other forms are so low that a quantitative change has affected a qualitative change in microsomal metabolism. In TCDD-treated adult rabbits, even though form 6 is induced, the increase is either too small or the background levels of the other forms are too high to establish a definite relationship between form 6 and benzo[a]pyrene metabolism. Because we do not know how the concentration of a particular form in microsomes is related to the rate of substrate turnover in those microsomes, we must rely on physical and immunological criteria for the identification of individual forms of cytochrome P-450 in crude preparations.

Studies in this and other laboratories with purified forms of cytochrome P-450 have provided electrophoretic, immunological, and physical criteria by which individual forms of cytochrome P-450 can be distinguished and identified. Our results demonstrate that particular forms of cytochrome P-450 can be identified in crude preparations by a direct comparison of the properties of purified forms with those of individual components in the crude preparation. Furthermore, the kinetic properties of microsomes reflect those of a specific cytochrome when that particular cytochrome form predominates. In view of the multiple forms of cytochrome P-450 in xenobiotic metabolism, variations in cytochrome composition during development and in response to inducers may have profound consequences on the susceptibility of an animal to drugs or chemical carcinogens. The development of criteria by which individual cytochrome P-450 forms can be identified and the application of these criteria to crude preparations are important steps toward our understanding of the role of individual forms of cytochrome P-450 in these processes.

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R L Norman, E F Johnson and U Muller-Eberhard


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