Induction of Two Immunocchemically Related Rat Liver Cytochrome P-450 Isozymes, Cytochromes P-450c and P-450d, by Structurally Diverse Xenobiotics*

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We have previously shown that purified rat liver cytochromes P-450c and P-450d share some but not all immunocchemical determinants (Reik, L. M., Levin, W., Ryan, D. E., and Thomas, P. E. (1982) J. Biol. Chem. 257, 3950-3957). Antibody to cytochrome P-450d cross-reacts with cytochrome P-450c to form an immuno precipitin band in the Ouchterlony test, but no detectable immunoprecipitin ring is formed in a radial immunodiffusion assay. However, the addition of purified cytochrome P-450c to purified cytochrome P-450d in the radial immunodiffusion assay alters the cytochrome P-450d standard curve. Appropriate corrections have been made for the interference of cytochrome P-450c in the immunoquantitation of cytochrome P-450d. Twelve structurally diverse xenobiotics have been examined for their capacity to modulate the levels of cytochrome P-450d, as well as cytochromes P-450a, P-450b, and P-450c, in rat liver microsomes. Five compounds (isosafrole, 3-methylcholanthrene, β-napthoflavone, 2,3,7,8-tetrachlorodibenzo-p-dioxin, and phenothiazine) and the polychlorinated biphenyl mixture Aroclor 1254 are potent in vivo inducers of cytochrome P-450d (0.44-0.89 nmol of cytochrome P-450d/mg of microsomal protein). Control rats have low levels of this microsomal hemoprotein (0.04-0.05 nmol of cytochrome P-450d/mg of microsomal protein). Isoasafrole induces cytochrome P-450d to a greater extent than cytochrome P-450c, Aroclor 1254 induces both hemoproteins to similar extents, and the remaining four compounds preferentially induce cytochrome P-450c relative to cytochrome P-450d. All of these structurally diverse compounds induce cytochromes P-450d and P-450c, suggesting that the inducibility of these cytochrome P-450 isoforms, but not cytochromes P-450a and P-450b, is linked.

Our laboratory has purified and characterized five hepatic microsomal cytochrome P-4501 isoforms from rats treated with different inducers of these hemoproteins. Cytochromes P-450a, P-450b, P-450c, P-450d, and P-450e can be considered isoforms since they appear to be products of different genes (1-8). The hepatic levels of cytochromes P-450a, P-450b, and P-450e have been shown to be under different regulatory control (7), whereas cytochromes P-450d and P-450b may be coordinately controlled. Until now, the regulation of cytochrome P-450d has not been investigated in detail. A comparison of the induction of cytochromes P-450d and P-450c is of particular interest since these hemoproteins have certain features in common. Although cytochromes P-450d and P-450c have different molecular weights, substrate specificities and peptide maps, as well as different NH₂- and COOH-terminal amino acid sequences, these hemoproteins are immunocchemically related and exhibit some similar spectral properties (2, 5, 9, 10). For example, these hemoproteins exhibit the same CO-reduced difference spectral peak at 447 nm, the reduced hemoproteins bind ethyl isocyanide with similar resultant 452:430 nm peak height ratios at pH 7.4, they both form an isosafrole metabolite-cytochrome P-450 complex with a single absorbance maximum at 455 nm (pH 7.4), and the ferrous hemoproteins do not form a 446 nm spectral complex with metyrapone (2, 3, 10). As a result of these similarities in the properties of cytochromes P-450d and P-450c, it is of interest to know whether these cytochromes are regulated in an independent or coordinate fashion. The results of the present study indicate that these hemoproteins are co-induced by structurally diverse xenobiotics but apparently are not under coordinate regulatory control.

EXPERIMENTAL PROCEDURES

Chemicals—β-Napthoflavone and phenothiazine were purchased from Aldrich Chemical Co., Inc. TCDD and 2-n-heptylbenezimidazole were gifts from Drs. A. Poland (McArdle Laboratories for Cancer Research, Madison, WI) and S. R. Challand (Wellcome Research Laboratories, London, England), respectively. Chlorpromazine HCl was purchased from Sigma Chemical Co. and isosafrole was purchased from Eastman Kodak Co. The sources for the remaining chemicals have been reported previously (7).

Xenobiotic Treatment of Rats and Preparation of Hepatic Microsomes—Immature male Long-Evans rats (50-60 g) from Blue Spruce Farms, Altamont, NY, were maintained on 12-h light cycles every 24 h in wire-bottom cages or on corn cob bedding and were allowed free access to water and Ratlson Purina Rodent Chow 5601. The following xenobiotics were administered by intraperitoneal injection on four different occasions.

1. G. P. Vlesnik, D. E. Ryan, P. E. Thomas, W. Levin, and F. G. Wats Jr. (1982) Biochemistry 21, 6288-6292.
2. The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; SD-SFAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the terms anti-P-450c, anti-P-450d, anti-P-4500(-d), and anti-P-4500(-c) refer to rabbit antibodies against rat liver microsomal cytochromes P-450c or P-4500 (cf. Ref. 9) which have been absorbed as described in "Experimental Procedures."
consecutive days and the rats were killed by decapitation 24 h after the last injection: 40 mg/kg/day of chlorpromazine HCl in water, 100 mg/kg/day of phenothiazine in corn oil, and 50 mg/kg/day of β-naphthoflavone in corn oil. TCDD was administered once by intraperitoneal injection at a dose of 10 µg/kg dissolved in dioxane (33.3 µg/ml) and the rats were killed on the fifth day. The dosing schedules for the other xenobiotics have been described (7). Immediately after decapsulation of the rats, the livers were removed and homogenized in 0.15 M KCl, 0.05 M Tris-HCl (pH 7.5 at 4 °C), and the microsomal fraction was isolated by differential centrifugation. The microsomes were washed by homogenization in 0.15 M KCl containing 10 mM EDTA, collected by centrifugation, suspended in 0.25 M sucrose, and stored at small aliquots at −76 °C until needed. When stored in this manner, without subsequent thawing and freezing, we have found no decrease in total cytochrome P-450 (as measured by CO-reduced difference spectra) or in cytochromes P-450b and P-450c (as measured by immunoquantitation) over the course of 2 years.

Total microsomal cytochrome P-450 was determined from the CO-reduced difference spectrum (11) in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. Prior to the spectral determination of cytochrome P-450 in microsomes from isosafrole-treated rats, the metabolic complex of cytochrome P-450 formed in vivo was displaced by incubation of microsomes with cytochrome c and n-heptylbenzimidazole (200 µM) for 20 min at 37 °C followed by 40 min at room temperature (12). The maximum final concentration of cytochrome P-450 during metabolite displacement was 5 µM, and the buffer used was the same as described above.

**Purification of Antigens and Immunochromatographic Procedures—Cytochromes P-450α, P-450b, and P-450c were purified to apparent homogeneity from Aroclor 1254-treated rats as described (1, 3). Cytochrome P-450αd was purified from isosafrole-treated rats (2). Quantitative single radial immunodiffusion assays were done with IgG against cytochromes P-450a, P-450b, P-450c, and P-450d. The preparation of antibodies against cytochromes P-450a, P-450b, and P-450c has been described (7, 13). Rabbit antisera against purified cytochrome P-450d inherently cross-react with cytochrome P-450c due to the immunochemical relatedness of these two hemoproteins (9). For this study, IgG was isolated from the antisera and immunoabsorbed against an ammonium sulfate-fractionated preparation of solubilized proteins from hepatic microsomes of phenobarbital-treated rats without subsequent thawing and freezing, we have found no decrease in total cytochrome P-450 (as measured by CO-reduced difference spectra) or in cytochromes P-450b and P-450c (as measured by immunoquantitation) over the course of 2 years.

**RESULTS**

**Immunodiffusion Studies with Anti-P-450d and Anti-P-450c—**The Ouchterlony double diffusion plate in Fig. 1A shows that anti-P-450d reacts with detergent-solubilized hepatic microsomes from rats treated with isosafrole to give one major and one minor immunoprecipitin band. Similarly, liver microsomes from rats treated with Aroclor 1254 or 3-methylcholanthrene also react with this antibody to give both a major and a minor immunoprecipitin band. The major immunoprecipitin bands formed between the antibody and the various microsomal preparations fuse with each other, showing that these microsomes contain an immunochemically identical antigen. When purified cytochrome P-450d is placed in at least two separate assays, and certain microsomal preparations were included in each experiment to verify reproducibility of the results.

**Fig. 1. Ouchterlony immunodiffusion analyses of microsomal samples with antibody to cytochrome P-450d.** The immunodiffusion plates in A and B were identical except for the omission of the deterrents Emulgen 911 (0.2%) and sodium cholate (0.5%) from B. The central wells (Anti-d) were filled with anti-P-450d at 10 mg of IgG/ml. Detergent-solubilized microsomes from rats treated with isosafrole (Iso Mx), Aroclor 1254 (Aro Mx), or 3-methylcholanthrene (MC Mx) were present at a final cytochrome P-450 concentration of 27, 26, and 48 μM, respectively. The wells labeled d contained 10 μM cytochrome P-450d. Unlabeled wells were filled with 0.9% NaCl. Wells in the agarose gel matrix were 4 mm in diameter.

present not only in liver microsomes from isosafrole-treated rats, the source from which it was purified, but also in liver microsomes from Aroclor 1254- or 3-methylcholanthrene-treated rats as previously discussed (9).

The minor immunoprecipitin band formed near the antibody well (Fig. 1A), when anti-P-450d reacts with solubilized microsomes, is not observed if purified cytochrome P-450d is tested against the antibody (Figs. 1 and 2). This weak immunoprecipitin band shows a reaction of immunochemical identity to the immunoprecipitin band formed by purified cytochrome P-450c (bottom well, Fig. 1A). Moreover, we have shown that absorption of anti-P-450d with a partially purified cytochrome P-450c preparation abolishes this weak secondary
immunoprecipitin band (9). By several additional criteria, including immunoaffinity chromatography and inhibition of catalytic activity, cytochromes P-450c and P-450d have been shown to be immunochemically related (9). The inherent immunchemical relatedness of cytochromes P-450c and P-450d has been established using both antibody to cytochrome P-450d and antibody directed against cytochrome P-450c, as shown in Fig. 2. Hybridoma antibodies directed against cytochrome P-450c have provided final proof for the immunochemical relatedness of these hemoproteins, since three of the 10 monoclonal antibodies cross-react strongly with cytochrome P-450d, whereas the remaining seven show no cross-reaction (15). In contrast, the apparent independent diffusion of the immunoprecipitin bands of cytochromes P-450c and P-450d (Fig. 1A) would, however, suggest these proteins are immunologically unrelated. We can only conclude that the Ouchterlony double diffusion test alone is insufficient for assignment of immunochemical relationships among these membrane-bound proteins (especially with weakly cross-reacting proteins).

The Ouchterlony double diffusion plate shown in Fig. 1B is identical with that in Fig. 1A except that sodium cholate and the nonionic detergent Emulgen 911 have been omitted from the agarose matrix. Clearly, the weak cross-reaction between cytochrome P-450c and anti-P-450d is greatly intensified when detergents are omitted from the double diffusion plate, indicating that detergents inhibit the reactivity of this antibody with the heterologous hemoprotein. However, the presence of detergents enhances the diffusion of the homologous antigen (i.e. cytochrome P-450d) in microsomal samples (compare the major immunoprecipitin bands in Fig. 1, A and B). The major immunoprecipitin bands exhibit less curvature and are displaced further from the antigen-containing wells in the presence of detergents (Fig. 1A) compared to these features in the absence of detergents (Fig. 1B).

Fig. 2, A and B, shows the reactivities of anti-P-450d and anti-P-450c with purified cytochromes P-450d and P-450c in the presence and absence of detergent in the Ouchterlony double diffusion plate. In Fig. 2A, when Emulgen 911 is present in the agarose matrix, the cross-reactions of anti-P-450c and anti-P-450d with the heterologous antigens are very weak. In Fig. 2B, when detergent is absent from the agarose matrix, the immunoprecipitin bands formed by cross-reacting proteins are intensified. Inclusion of the detergent facilitates diffusion of the homologous purified antigen as shown by the diminished curvature of the immunoprecipitin bands in Fig. 2A compared to the corresponding immunoprecipitin bands in Fig. 2B. Consequently, two effects of detergent in the immunodiffusion media are apparent: 1) detergent facilitates diffusion of detergent-solubilized microsomal samples and to a lesser extent facilitates diffusion of the purified antigens; and 2) detergent partially inhibits immunoprecipitation, especially evident with weakly cross-reacting antigens. We have recently described a very similar detergent effect in Ouchterlony double diffusion studies with human hepatic microsomal epoxide hydrolase and its antibody (16). In that study, the cross-reacting protein was epoxide hydrolase from different mammalian species.

Radial Immunodiffusion Assay of Cytochrome P-450d—As discussed above, we have previously shown that anti-P-450d can be absorbed against partially purified cytochrome P-450c to remove cross-reacting antibodies (9). The resulting antibody preparation (anti-P-450d(−c)) was used in our initial attempts to quantitate levels of cytochrome P-450d in rat liver microsomes. Anti-P-450d(−c) proved to be ineffective in the radial immunodiffusion assay, yielding irreproducible immunoprecipitin rings as well as a nonproportional relationship between cytochrome P-450d concentration and the square of the immunoprecipitin ring diameter. We speculated that the poor quality of anti-P-450d(−c) in the radial immunodiffusion assay might be the result of insufficient amounts of antibody, or poor affinity antibody, directed against antigenic determinants unique to cytochrome P-450d. We therefore decided to try anti-P-450d, i.e. the antibody to cytochrome P-450d which inherently cross-reacts with cytochrome P-450c, in the radial immunodiffusion assay. Fig. 3A shows the results of a radial immunodiffusion assay where anti-P-450d was uniformly distributed in the agarose, and the square of the precipitin ring diameter minus the square of the well diameter (d'2 − 6.25) is plotted against the concentration of purified cytochrome P-450d. This relationship is a linear function except at low concentrations of cytochrome P-450d (below 0.5 μM) where a
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FIG. 3. The effect of cytochrome P-450c on the radial immunodiffusion assay of purified cytochrome P-450d and the dependence of the cytochrome P-450d assay correction factor on the molar ratio of cytochrome P-450c to P-450d. A shows the effect of various concentrations of cytochrome P-450d on the square of the immunoprecipitin ring diameter, and the contribution of a 3.9-fold molar excess of purified cytochrome P-450c to P-450d on the cytochrome P-450d standard curve. B graphically presents the dependence of the cytochrome P-450d assay correction factor on increasing molar ratios of cytochrome P-450c to P-450d. A product of the appropriate correction factor with the percentage of immunoassayed microsomal cytochrome P-450d compensates for the contribution of cytochrome P-450c in the assay of cytochrome P-450d.

departure from linearity is observed. A practical result of this phenomenon is that microsomal samples containing less than 5% of the total cytochrome P-450 as cytochrome P-450d are measured with less accuracy because they fall in the nonlinear region of the standard curve for cytochrome P-450d. It was also determined that the best linear relationship between concentration of cytochrome P-450d and ring diameter (d² − 6.25) was obtained when detergents were included in the agarose matrix, as previously described (7), especially in the case of solubilized microsomes (data not shown).

As can also be seen in Fig. 3A, the standard curve for cytochrome P-450d is shifted to larger ring diameters when purified cytochrome P-450c is mixed with cytochrome P-450d, even though purified cytochrome P-450c by itself gives no detectable immunoprecipitin ring. When low concentrations of solubilized microsomes from untreated rats, which contain only small amounts of cytochromes P-450c and P-450d, or purified cytochrome P-450b, are mixed with purified cytochrome P-450d, there is no significant effect on the standard curve for cytochrome P-450d (data not shown). Obviously, the effect of cytochrome P-450c on the reaction of cytochrome P-450d in the radial immunodiffusion assay is a consequence of shared immunochemical determinants. The effect of various amounts of purified cytochrome P-450c on the reaction of purified cytochrome P-450d with anti-P-450d versus the appropriate correction factor is plotted in Fig. 3B. After first quantitating cytochrome P-450d in microsomes with anti-P-450c and calculating the ratio of cytochrome P-450c to cytochrome P-450d, the appropriate correction factor was applied to the percentage of total microsomal cytochrome P-450 which is present as cytochrome P-450d to yield the corrected value for cytochrome P-450d in microsomes. It is fortuitous that the correction factor and the molar ratio of cytochrome P-450c to P-450d are linearly related over most of the ratios obtained with liver microsomes. In practice, only microsomes from untreated and corn oil-treated rats appeared to have a cytochrome P-450c to P-450d ratio lower than 0.4, but no correction was attempted since both proteins are present at very low concentrations in these microsomes (cf. Table I).

Cytochrome P-450d purified from isosafrole-treated rats still contains an isosafrole metabolite bound to the heme moiety (2). Displacement of this metabolite by either cyclohexane or 2-n-heptylbenzimidazolone has no effect on the reaction of cytochrome P-450d in the radial immunodiffusion assay. In addition, anti-P-450d prepared against cytochrome P-450d with or without metabolite bound to the hemoprotein shows no difference in its reaction with the purified cytochrome. These results indicate that this isosafrole metabolite-cytochrome P-450d complex has no apparent effect on the immunochemical recognition of cytochrome P-450d.

Immunochemical Quantitation of Cytochromes P-450a, P-450b, P-450c, and P-450d in Liver Microsomes from Immature Male Rats Treated with Various Xenobiotics—Hepatic drug metabolism has long been known to be markedly affected by xenobiotic treatment of animals (7) but, in the absence of assays specific for individual cytochromes (7, 13), these effects could not be correlated with the levels of individual forms of cytochrome P-450. Antibodies specific for each of several cytochrome P-450 isozymes have recently been shown to provide sufficient specificity to quantitate these proteins in liver microsomes (7, 13). These results have established the selective influence of age, sex, and induction by several xenobiotics on the levels of cytochromes P-450a, P-450b, and P-450c in rat hepatic microsomal preparations (7, 13). Table I lists the level of cytochrome P-450d, as well as cytochromes P-450a, P-450b, and P-450c, in hepatic microsomes from rats treated with 12 different xenobiotics known to alter microsomal drug-metabolizing capacity. The values for cytochrome P-450d were corrected for any contribution of cytochrome P-450c, as shown in Fig. 3, and described above. The amount of each isozyme is expressed as a percentage of the total spectrally determined microsomal cytochrome P-450 and as nanomoles of each isozyme per mg of microsomal protein (values in parentheses).

Recent studies have shown that the quantitation of cytochrome P-450b necessarily includes cytochrome P-450a since these hemoproteins are immunochemically indistinguishable in Ouchterlony double diffusion analysis (3, 18) and in radial immunodiffusion when tested against antibody to cytochrome P-450b (results not shown). In our first immunoquantitation study in 1979 (13), we recognized the possibility that additional uncharacterized forms of cytochrome P-450 could cross-react with antibodies directed against characterized forms and therefore contribute to immunoquantitation of a given isozyme. The cross-reacting protein would be overlooked if it had not been previously purified and if it had a mobility in SDS-PAGE very similar to the antigen of immunization. Cytochrome P-450c has only recently been purified and has a
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Relative increase in levels of cytochromes P-450d and P-450c, also induce cytochromes P-450b. All of the compounds tested that are potent inducers of other compounds may decrease the levels of these isozymes. Interestingly, each compound that is an effective inducer of phenothiazine, chlorpromazine, or Aroclor 1254 results in marked induction of hepatic microsomal cytochrome P-450d.

Isosafrole induces cytochrome P-450d in rat liver to the greatest extent, compared to the other xenobiotics tested, yielding an 18-fold increase in the specific content of this isozyme. Interestingly, each compound that is an effective inducer of cytochrome P-450d is also a significant inducer of cytochrome P-450c, with the possible exception of chlorpromazine, also induce cytochrome P-450a. Those xenobiotics that do not induce cytochromes P-450b and P-450c (i.e., 3-methylcholanthrene, β-naphthoflavone, TCDD) are marginally more effective inducers of cytochrome P-450a.

Phenothiazine, chlorpromazine, or Aroclor 1254 results in marked induction of hepatic microsomal cytochrome P-450d. Isosafrole induces cytochrome P-450d in rat liver to the greatest extent, compared to the other xenobiotics tested, yielding an 18-fold increase in the specific content of this isozyme. Interestingly, each compound that is an effective inducer of cytochrome P-450d is also a significant inducer of cytochrome P-450c, with the possible exception of chlorpromazine, also induce cytochrome P-450a. Those xenobiotics that do not induce cytochromes P-450b and P-450c (i.e., 3-methylcholanthrene, β-naphthoflavone, TCDD) are marginally more effective inducers of cytochrome P-450a.

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Table I

| Rat treatment | Total cytochrome P-450 (nmol/mg protein) | Per cent of total cytochrome P-450 |
|--------------|------------------------------------------|----------------------------------|
| Untreated (n = 4) | 0.86 ± 0.04 (0.05) | P-450a 6 (0.20) | P-450b + P-450c 4 (0.03) | P-450d 5 (0.37) | Unknown 1 (0.10) |
| Corn oil (n = 7) | 0.96 ± 0.22 (0.07) | P-450a 5 (0.02) | P-450b + P-450c 3 (0.01) | P-450d 1 (0.05) | Unknown 1 (0.06) |
| Isosafrole (n = 5) | 2.33 ± 0.23 (0.12) | P-450a 1 (0.40) | P-450b + P-450c 1 (0.37) | P-450d 1 (0.89) | Unknown 1 (<0.02) |
| 3-Methylcholanthrene (n = 7) | 1.85 ± 0.10 (0.26) | P-450a 1 (0.02) | P-450b + P-450c 1 (1.34) | P-450d 0.44 |
| β-Naphthoflavone | 3.67 ± 1.57 (0.14) | P-450a 26 (0.62) | P-450b + P-450c 1 (1.12) | P-450d 0.60 |
| Chlorpromazine | 1.96 ± 0.09 (0.08) | P-450a 9 (0.20) | P-450b + P-450c 1 (0.31) | P-450d 0.51 |
| Aroclor 1254 (n = 2) | 0.86 ± 0.05 (0.03) | P-450a 7 (0.20) | P-450b + P-450c 27 (0.99) | P-450d 0.81 |
| Phenobarbital (n = 2) | 1.85 ± 0.67 (0.26) | P-450a 5 (1.36) | P-450b + P-450c 1 (0.92) | P-450d 0.81 |
| γ-Chlordane | 2.18 ± 0.55 (0.13) | P-450a 6 (1.20) | P-450b + P-450c 1 (0.92) | P-450d 0.81 |
| SKF-525A | 1.88 ± 0.88 (0.08) | P-450a 4 (0.87) | P-450b + P-450c 2 (0.06) | P-450d 0.04 |
| trans-Stilbene oxide | 2.26 ± 0.35 (0.09) | P-450a 4 (0.81) | P-450b + P-450c 1 (0.09) | P-450d 0.02 |
| Pregnenolone-16α-carbonitride (n = 3) | 1.57 ± 0.15 (0.06) | P-450a 4 (0.75) | P-450b + P-450c 2 (0.03) | P-450d 0.01 |

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450 (0.72-0.86 nmol/mg of protein). Treatment of rats with potent inducers of cytochrome P-450c, however, results in a dramatic reduction in the specific content of the unknown fraction of cytochrome P-450, suggesting that the major forms of cytochrome P-450 present in control rats may be decreased by inducers of cytochrome P-450c. Previous results of SDS-PAGE and two-dimensional gel electrophoresis have also provided support for this concept (19, 20).

In Table I, the sum of cytochromes P-450a, P-450b + P-450c, P-450d, and P-450d total greater than 100% of microsomal cytochrome P-450 (determined spectrally) following treatment of rats with certain inducers such as 3-methylcholanthrene. There are several possible factors contributing to this problem: (a) the total microsomal cytochrome P-450 values do not include cytochrome P-420; (b) spectrally determined cytochrome P-450 does not include any apocytochrome P-450; (c) other forms of cytochrome P-450, not yet identified, could cross-react with some of the antibodies, although no evidence has been obtained for this proposal; (d) the purified cytochrome P-450 standards may not diffuse and react with an antibody in a fashion identical with the hemoproteins in detergent-solubilized microsomes; and (e) the error in determination of unknown cytochrome P-450 is greater than the error in determination of any single cytochrome P-450, since the unknown value is derived from a composite of the five isozymes.

We have not included cytochrome P-420 as part of the total cytochrome P-450 content of liver microsomes since the spectral estimation of cytochrome P-420 is not precise in the presence of an excess of cytochrome P-450. However, in contrast to the purified cytochromes, most microsomal preparations contain at least 10-15% of the cytochrome P-450 content as cytochrome P-420. Ultimately, only further investigation of each of these factors will resolve this problem. Nevertheless, the relative proportions of these five isozymes of cytochrome P-450 does seem to be consistent with a variety of data derived from purification, SDS-PAGE, two-dimensional gel electrophoresis, and catalytic activity studies (1-3, 9, 18).

Table II presents data exploring the effects of combined 3-methylcholanthrene and isosafrole treatment on the induction of microsomal cytochromes P-450b + P-450c, P-450d, and P-450d in rat liver. Fennell et al. (21) originally examined the combined effects of these inducers on rat liver microsomal cytochrome P-450 by SDS-PAGE and spectral determinations of the isosafrole metabolite-cytochrome P-450 complex. Treatment of rats with 3-methylcholanthrene for 3 days followed by corn oil for 1 day has approximately the same inductive effect on cytochromes P-450c and P-450d as does 4 consecutive days of 3-methylcholanthrene treatment (compare data in Table II with those in Table I). In both of these instances, cytochromes P-450c and P-450d constitute 78-79% and 17-24%, respectively, of the total microsomal cytochrome P-450.

One administration of isosafrole to rats after 3 days of corn oil results in an induction of cytochrome P-450d that is approximately equivalent to the induction of this hemoprotein observed following 3-methylcholanthrene treatment; in contrast, the levels of cytochrome P-450c differ dramatically following 3-methylcholanthrene treatment compared to a single isosafrole treatment. When isosafrole is administered only once, the specific content of cytochrome P-450d (0.39 nmol/mg of protein) is only 32% of that obtained when rats were treated with isosafrole for 4 days (1.21 nmol of cytochrome P-450d/mg of protein). The specific content of cytochrome P-450c, however, increases less than 2-fold when the value obtained for this hemoprotein after one injection (0.37 nmol/mg of protein) is compared to the value obtained after four injections of isosafrole (0.61 nmol/mg of protein). When 3-methylcholanthrene

| Treatment          | Days of treatment | Total cytochrome P-450 | P-450b + P-450c | P-450c | P-450d |
|--------------------|-------------------|-----------------------|-----------------|--------|--------|
| Corn oil           | 4                 | 0.81                  | 1               | 5      |        |
| Isosafrole         | 4                 | 2.89                  | 19              | 21     | 42     |
| 3-Methylcholanthrene| 3                 | 1.89                  | 1               | 79     | 17     |
| Corn oil           | 1                 | 0.02                  | 0.55            | 0.61   | 1.21   |
| Isosafrole         | 3                 | 3.64                  | 11              | 39     | 50     |
| Corn oil           | 3                 | 1.85                  | 28              | 20     | 21     |
| Isosafrole         | 1                 | 0.52                  | 0.32            | 1.42   | 1.82   |

Immature male rats were injected intraperitoneally once each day with the compounds listed, and killed 24 h after the last injection (25 mg/kg/day for 3-methylcholanthrene and 150 mg/kg/day for isosafrole). When both compounds were administered to rats, they were given at a 24-h interval between compounds, in the order given in the first column. Numbers in parentheses give the amount of the cytochrome P-450 isozyme in nanomoles/mg of microsomal protein. The legend for Table I should be consulted for details not listed here.

The SDS-gel electrophoretic profiles of rat liver microsomes shown in Fig. 4 are consistent with immunooquantitative determinations of the various cytochrome P-450 isozymes. Microsomes from corn oil-treated rats have no detectable, discrete protein-staining bands of the same mobility as purified cytochromes P-450c (M, = 56,000), or P-450b and P-450d (M, = 52,000), which illustrates the very low levels of these proteins as measured by immunonquantitation (Tables I and II). The staining intensity of a microsomal protein that co-migrates with purified cytochrome P-450c is greatest after treatment of rats with 3-methylcholanthrene or 3-methylcholanthrene plus isosafrole. This protein is also induced, but to a lesser extent, after treatment of rats with isosafrole alone. The relative staining intensity of the M, = 56,000 protein band in microsomes is consistent with the levels of cytochrome P-450c determined by immunonquantitation (Table II). Although cytochromes P-450b and P-450d co-migrate in this SDS-PAGE system, the microsomal protein-staining band that contains these two proteins is increased to the greatest extent after the combined 3-methylcholanthrene/isosafrole treatment of rats (compare values in Table II with Fig. 4). Isosafrole treatment of rats for 4 days results in the second highest concentration...
The percentage of cytochrome P-450 present in microsomes complexed with an isosafrole metabolite was estimated from the difference in spectrally measurable cytochrome P-450 before and after treatment of the microsomes with the displacer cyclohexane. Cyclohexane (10 mM final concentration) has previously been reported to dissociate effectively the isosafrole metabolite from the heme of cytochrome P-450 (12), thereby eliminating the inhibition of carbon monoxide binding caused by the metabolite. As listed in Table III, 33–41% of the total liver microsomal cytochrome P-450 from rats treated for 4 days with isosafrole is present as a complex with an isosafrole metabolite. Interestingly, approximately the same percentage of metabolite-bound cytochrome P-450 is present in liver microsomes from rats treated once with isosafrole subsequent to 3 days of 3-methylcholanthrene pretreatment. If only one injection of isosafrole is administered to rats, only 17% of the total microsomal cytochrome P-450 is bound to an isosafrole metabolite. The amount of cytochrome P-450d in these microsomal preparations determined immunoenzymographically parallels the amount of complexed cytochrome P-450. These results (Table III) clearly indicate a marked selectivity in binding of an isosafrole metabolite to cytochrome P-450d \textit{in vivo}. These data are consistent with our previous observations that cytochromes P-450a, P-450b, and P-450c purified from isosafrole-treated rats do not have an isosafrole metabolite bound to the heme moiety, even though purified cytochromes P-450b and P-450c do form this hemoprotein-isoafrole metabolite complex \textit{in vitro} (2). Although these results suggest that the amount of cytochrome P-450d in microsomes from isosafrole-treated rats can be approximated by measurement of the isosafrole metabolite complex, we cannot rule out the \textit{in vivo} formation of this complex with other minor forms of cytochrome P-450 in these microsomes.

**DISCUSSION**

The ability to quantitate each isozyme of cytochrome P-450 in microsomal membranes would have a major impact on our understanding of the effects of xenobiotics on cytochrome P-450 activity. Table III shows a comparison between the levels of cytochrome P-450d determined by immunoquantitation and the amount of spectrally measured isosafrole metabolite-cytochrome P-450 complex in the same microsomal preparations following treatment of rats with isosafrole.

![Fig. 4. SDS-PAGE profile of liver microsomes from rats treated with xenobiotics as described in Table II. Purified cytochromes P-450c and P-450d (0.4 μg each) were placed in wells on the extreme left and right of the microsomal samples. Cytochromes P-450d and P-450b are not resolved in this SDS-PAGE system (cf. Ref. 2) and the lower standard is labeled P-450b/d to indicate this fact. Microsomal protein (6 μg) was applied to the gel and C, I, and MC refer to rat treatments with corn oil, isosafrole, or 3-methylcholanthrene, respectively. The numbers in parentheses refer to the number of days of xenobiotic treatment as described in Table II.]

![Table III](http://www.jbc.org/Downloaded from)

| Treatment          | Days of treatment | Total cytochrome P-450 | Total cytochrome P-450 as a metabolite complex | Total cytochrome P-450 as isosafrole metabolite complex |
|--------------------|-------------------|------------------------|-----------------------------------------------|--------------------------------------------------------|
| Isosafrole         | 4                 | 2.36                   | 37                                            | 39                                                     |
| Isosafrole         | 4                 | 1.92                   | 35                                            | 41                                                     |
| Isosafrole         | 4                 | 2.80                   | 37                                            | 33                                                     |
| Isosafrole         | 4                 | 1.70                   | 41                                            | 39                                                     |
| Isosafrole         | 4                 | 2.89                   | 42                                            | 36                                                     |
| 3-Methylcholanthrene | 1              | 3.64                   | 50                                            | 40                                                     |
| Isosafrole         | 1                 | 1.85                   | 21                                            | 17                                                     |

The ability to quantitate each isozyme of cytochrome P-450 in microsomal membranes would have a major impact on our understanding of the effects of xenobiotics on cytochrome P-450 activity.
understanding of the regulation of this unique family of hemoproteins. Unfortunately, classical techniques such as spectral determinations, substrate specificity, or the use of specific inhibitors have not provided the high degree of specificity required to make such determinations. The use of antibodies directed against individual isozymes has held great promise in this area but, like all other methods applied to these hemoproteins, certain problems have arisen. Rat liver cytochromes P-450a, P-450b, and P-450c are immunologically distinct from one another and were the first three isozymes to be immunquantitated in microsomal preparations (7, 13). The effects of age, sex, and, in particular, xenobiotic treatment of rats, have been shown to markedly alter both the absolute and relative amounts of these isozymes in liver microsomes (7). Several different immunquantitative techniques (23–25) have since been applied to two of these isozymes (cytochromes P-450b and P-450c), and the results are in good agreement with our original observations (13). However, when cytochrome P-450d was purified from isosafrole-treated rats, it was found to cross-react weakly with antibody prepared against cytochrome P-450c. These two proteins (cytochrome P-450c and P-450d) have since been shown to share some immunological determinants (9, 15), and the cross-reacting antibodies can be removed by absorption of the antibody preparations with the heterologous antigen (9). However, the weak cross-reaction of cytochrome P-450d with anti-P-450c does not interfere in the immunquantitation of cytochrome P-450c as determined by the radial immunodiffusion assay (9). Presumably, the immunological cross-reactivity of cytochromes P-450c and P-450d could be a major problem in a radioimmunoassay or an enzyme-linked immunosorbent assay without appropriate absorption of the antibody since only one common antigenic determinant is required for detection in these assays. From our experience, the cross-reaction of cytochrome P-450c with anti-P-450d is not a limited observation since it has been seen in all immunized animals examined to date (three sheep, two mice, and 21 rabbits).

When anti-P-450d was prepared and shown to cross-react with cytochrome P-450c, it appeared to us that an effective approach to eliminate any potential problem in the immunquantitation of cytochrome P-450d would be to absorb out the cross-reacting antibodies against cytochrome P-450c, as previously described (9). Unfortunately, results obtained with this antibody preparation (anti-P-450d(–c)) were variable, and barely detectable immunoprecipitin rings were formed in the radial immunodiffusion assay. When anti-P-450d that cross-reacts with cytochrome P-450c was tested in this assay system, it was found to give highly reproducible immunoprecipitin rings which could easily be used for the quantitation of cytochrome P-450d. Unfortunately, cytochrome P-450c, which does not form immunoprecipitin rings with this antibody, does interfere in the quantitation of cytochrome P-450d. This problem was overcome by generating a standard curve of various ratios of purified cytochromes P-450c to P-450d and correcting for the contribution of cytochrome P-450c in microsomes. No other microsomal proteins examined (i.e., solubilized microsomes from control rats, which contain very low levels of cytochromes P-450c and P-450d, and purified cytochrome P-450b) have any effect on the immunquantitation of cytochrome P-450d when they are added to the purified standard. Furthermore, anti-P-450d does not appear to react with microsomal proteins other than cytochromes P-450d and P-450c when analyzed by Ouchterlony double diffusion analysis, quantitative radial immunodiffusion, or immunoaffinity chromatography.

Immunquantitation of cytochrome P-450d in liver microsomes has revealed that this hemoprotein is present in very low amounts in control rats (0.04–0.05 nmol/mg of microsomal protein) and is induced 18-fold by isosafrole (0.89 nmol/mg of protein). Interestingly, all compounds (or mixtures of compounds) that we found to be inducers of cytochrome P-450d are also inducers of cytochrome P-450c. These include 3-methylcholanthrene, the polychlorinated biphenyl mixture Aroclor 1254, TCDD, β-naphthoflavone, phenothiazine, chlorpromazine, and isosafrole. These results suggest that although cytochromes P-450c and P-450d are co-induced in these hemoproteins are apparently not under coordinate control since the ratio of cytochrome P-450c to P-450d in microsomes varies from 0.4 in isosafrole-treated rats to 3.2 in microsomes from 3-methylcholanthrene-treated rats. Most of these compounds also result in a 2- to 4-fold increase in cytochrome P-450a with the possible exception of chlorpromazine. Thus, at least three cytochrome P-450 isozymes are inducible by the classical “MC-type inducers.” Since the inducibility of these three cytochrome P-450 isozymes is not under coordinate regulatory control, it may be possible to induce one isozyme without affecting the other two isozymes. The induction of cytochrome P-450c by phenothiazine and chlorpromazine is the likely explanation for the observed induction of benz(a)pyrene metabolism in rat liver following treatment of rats with these compounds (26). Benz(a)pyrene is most efficiently metabolized by cytochrome P-450c compared to cytochromes P-450a, P-450b, P-450d, and P-450e (1–3). Interestingly, isosafrole, chlorpromazine, and phenothiazine but not 3-methylcholanthrene, β-naphthoflavone, or TCDD, increase the major phenobarbital-inducible cytochromes P-450 (P-450b + P-450e) in rat liver microsomes. However, phenobarbital, γ-chlordane, SKF-525A, and trans-stilbene oxide, which are potent inducers of cytochromes P-450b + P-450e, do not induce cytochrome P-450c or P-450d.

The co-induction of cytochromes P-450c and P-450d by certain xenobiatics, as measured by radial immunodiffusion, has been verified by several additional observations. First, we previously described the purification of both cytochromes P-450c and P-450d from isosafrole-treated rats (2). The very low concentrations of cytochromes P-450c and P-450d in livers of control or phenobarbital-treated rats preclude the purification of the hemoproteins using our present methodology. Second, results of immunoaffinity chromatography with antibodies covalently bound to Sepharose have shown that both proteins are present in elevated levels in microsomal 3-methylcholanthrene- and isosafrole-treated rats (9, 13). Third, Ouchterlony double diffusion analysis with anti-P-450c(–d) or anti-P-450d has indicated that these proteins are elevated in microsomes from rats treated with Aroclor 1254, isosafrole, or 3-methylcholanthrene but not in rats treated with phenobarbital (cf. Fig. 1). Finally, two-dimensional isoelectric focusing-SDS-PAGE has shown that protein-staining bands co-migrating with purified cytochromes P-450c and P-450d are markedly increased in liver microsomes of rats treated with isosafrole, 3-methylcholanthrene, or Aroclor 1254 but not in microsomes from rats treated with phenobarbital, γ-chlordane, SKF-525A, trans-stilbene oxide, or pregnenolone-16α-carbonitrile.

Other investigators have presented results of SDS-PAGE of rat liver microsomes alluding to the presence of two major hemoproteins induced by 3-methylcholanthrene or TCDD with minimum molecular weights approximating purified cytochromes P-450c and P-450d (19, 27, 28). However, interpretation of changes in intensity of protein-staining bands in SDS-PAGE of microsomes is fraught with difficulties that are...
greatly diminished in two-dimensional isoelectric focusing-SDS-PAGE. For example, several non-cytochrome P-450 proteins have minimum molecular weights in the 48,000–60,000 range, although they differ in isoelectric points, and several forms of rat liver cytochrome P-450 are poorly resolved in one-dimensional SDS-PAGE (cytochromes P-450b, P-450c, and P-450d) but clearly separate in the isoelectric focusing dimension of the two-dimensional gel system of Vlasuk and co-workers (18, 20). It is also known that certain cytochrome P-450 isozymes which are not resolved in SDS-PAGE are induced by isosafrole, phenothiazine, chlorpromazine, or Aroclor 1254 treatment of rats, and hence, one protein-staining band contains at least two proteins. Nevertheless, the results of SDS-PAGE presented elsewhere (2, 9, 19, 27, 28) and in the present study are consistent with the induction of cytochromes P-450c and P-450d by 3-methylcholanthrene or isosafrole treatment of rats.

While antibody specificity for various cytochrome P-450 isozymes is, unfortunately, not absolute, the degree of specificity is sufficient to analyze microsomes for a number of these isozymes in the presence of other forms. This approach to understanding and studying the regulation of cytochrome P-450 induction is certainly far superior to other methodologies available at the present time. In particular, anti-P-450b and anti-P-450c–(d) have been used to selectively immunoprecipitate in vitro cell-free translation products (29–34). These experiments have led to the successful cloning of cDNA coding for these proteins (35–37), and the total sequence of a rat liver cytochrome P-450 has recently been reported (38).

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