Estimation of Isozymes of Microsomal Cytochrome P-450 in Rats, Rabbits, and Humans Using Immunochemical Staining Coupled with Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis†

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ABSTRACT: Microsomal proteins were resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and transferred to sheets of nitrocellulose. Specific forms of cytochrome P-450 (P-450) were identified on the sheets with the use of an immunoperoxidase staining technique and rabbit antibodies raised to electrophoretically homogeneous forms of rat and human liver P-450. The amounts of each form of P-450 present in microsomal preparations could be detected and quantitated by densitometry at the picomole level. A form of P-450 denoted P-450 PB-B_2 accounted for the majority of the P-450 present in liver microsomes of rats treated with phenobarbital, *trans*-stilbene oxide, or dimethylnitrosamine. A similar protein was detected in rat lung microsomes regardless of treatment. Another form of P-450 denoted P-450 BNF-B_2 accounted for the majority of the P-450 present in liver microsomes of rats treated with 5,6-benzoflavone, 3-methylcholanthrene, or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. A similar protein was induced by 5,6-benzoflavone in rat lung and kidney. Both forms of P-450 were induced in rat liver by the polychlorinated biphenyl mixture Aroclor 1254. Detectable levels of P-450s resembling these two forms were very low in livers of untreated rats, mice, and rabbits, in livers of rats treated with 2-(acetylamino)-fluorene or pregnenolone-16α-carbonitrile, and in rat hearts and brains. Antibodies raised to liver P-450s reacted with the inducible rabbit liver P-450s denoted P-450 LM-2 and P-450 LM-4 and were used to quantitate induction of those proteins. Antibodies raised to human liver P-450 purified from a single patient recognized a protein with identical electrophoretic mobility in liver microsomes prepared from ten different patients and also recognized a protein with a higher apparent monomeric molecular weight in the lung microsomes of two of these patients examined. The portion of total human liver microsomal P-450 that reacted with the antibody varied from 6% to 56% among ten different patients. The sensitivity and specificity of these techniques may be of further use in the study of P-450 multiplicity.

A microsomal mixed-function oxidase system containing cytochrome P-450[1] functions in the metabolism of a wide variety of endogenous and xenobiotic compounds in many mammalian tissues. The various P-450 isozymes are induced by different chemicals and can metabolize a number of substrates in different ways (Lu & West, 1980).

The identification and quantitation of individual forms of P-450 in crude mixtures has been a problem that has inhibited the understanding of P-450 multiplicity. Different forms of P-450 are distinguished by substrate specificity (Lu & West, 1980; Guengerich, 1979) and regio- and stereoselectivity toward individual substrates (Ryan et al., 1979; Kaminsky et al., 1980), but significant overlap exists in many cases (Johnson, 1979). Spectral differences are also overlapping (Guengerich, 1979; Johnson, 1979). While individual forms of P-450 can be isolated in apparently homogeneous form, yields are considerably less than quantitative (Coon et al., 1978; Guengerich, 1979). Ion-exchange methods have been developed that permit partial separation of P-450s with high recovery (Warner et al., 1978; Kotake & Funae, 1980), but these procedures have not been shown to distinguish single electrophoretic forms of P-450. In recent years a number of investigators have raised antibodies to highly purified P-450 preparations and used these preparations to study various forms of P-450 in crude mixtures (Lu & West, 1980; Guengerich, 1979; Ryan et al., 1979; Johnson, 1979; Welton et al., 1975; Kamataki et al., 1976; Thomas et al., 1976a; Dean & Coon, 1977; Elshourbagy & Guzelian, 1980; Guengerich & Mason, 1979; Masuda-Mikawa et al., 1979; Serabjit-Singh et al., 1979; Tsuji et al., 1980; Kaminsky et al., 1979, 1980, 1981; Dus et al., 1980). Most of these antibody preparations have been shown to recognize forms of P-450 other than the antigen used for immunization. Some investigators have used adsorption methods to remove cross-reactive populations of antibodies (Thomas et al., 1979, 1981), but such preparations still recognize more than one form of P-450 (Ryan et al., 1979, 1980; Ryan & Levin, 1981).

The usefulness of sodium dodecyl sulfate–polyacrylamide gel electrophoresis in separating individual forms of P-450 as a function of apparent monomer molecular weight has been recognized for some time (Lu & West, 1980; Guengerich, 1979; Welton & Aust, 1974; Haugen et al., 1975). However, positive assignment of resolved bands to individual forms of P-450 has been hindered by the abundance of microsomal proteins migrating in the 45 000–60 000-dalton region and the

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[1] Abbreviations: P-450, microsomal cytochrome P-450; PB, phenobarbital; BNF, β-naphthoflavone (5,6-benzoflavone); 3MC, 3-methylcholanthrene; AAF, 2-(acetylamino)fluorene; SO, *trans*-stilbene oxide; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCN, pregnenolone-16α-carbonitrile; DMN, dimethylnitrosamine; IgG, immunoglobulin G; PBS, phosphate-buffered saline [20 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl]; ip, intraperitoneally; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; P-450 PB-B_2, the major form of P-450 isolated from liver microsomes of PB-treated rats (Guengerich & Martin, 1980); P-450 BNF-B_2, the major form of P-450 isolated from liver microsomes of BNF-treated rats (Guengerich & Martin, 1980); P-450 LM-2, the major form of P-450 isolated from liver microsomes of PB-treated rabbits (Coon et al., 1978); P-450 LM-4, the major form of P-450 isolated from liver microsomes of BNF-treated rabbits (Coon et al., 1978).

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tendency of the heme to leave P-450s (Welton & Aust, 1974; Haugen et al., 1975) and bind to other proteins (Thomas et al., 1976b). We have attempted to use a number of immunological techniques to identify individual forms of P-450 resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, including direct incubation of unlabeled and fluorescent antibodies with polyacrylamide gels (Olden & Yamada, 1977), crossed-rocket immunoelectrophoresis (Chua & Blomberg, 1979), and transfer of proteins from polyacrylamide to diazotized papers (Renart et al., 1979), but success has been variable with all of these techniques. We have now taken advantage of the nitrocellulose transfer technique of Towbin et al. (1979), an immunoperoxidase staining technique developed by Glass et al. (1981), and the availability of highly specific antisera raised to two distinct forms of rat liver P-450 (Guengerich & Martin, 1980; Guengerich, 1977b, 1978a) and one form of human liver P-450 (Kaminsky et al., 1981; Guengerich et al., 1981) to study the induction and variation of P-450s in different tissues of rats, rabbits, mice, and humans. The techniques used permit the identification of individual forms of P-450 by apparent monomer molecular weight and immunological reactivity, two of the most sensitive probes of P-450 differences.

Experimental Procedures

Chemicals. Goat anti-rabbit IgG was purchased from either Miles Chemical Co. (Elkhart, IN), Boehringer-Mannheim (Indianapolis, IN), or Serasource, Inc. (Berlin, MA). Horseradish peroxidase–rabbit anti-horseradish peroxidase complex was also purchased from Miles. 3,3′-Diaminobenzidine hydrochloride and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose filters (0.45 μm; catalog no. HAWP 304 FO) were obtained from Millipore Corp. (Bedford, MA). Calf serum was obtained through Grand Island Biologicals Co. (Grand Island, NY). PCN was a gift of Dr. P. O'Connell of the Upjohn Co. (Kalamazoo, MI). Aroclor 1254 was obtained from Analabs (North Haven, CT). TCDD was a gift of the Dow Chemical Co. (Midland, MI). SO, AAF, DMN, and BNF were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Reagents for gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). All water used was purified by deionization and subsequent passage through a Milli-Q filtration apparatus (Millipore Corp.). Other reagents were of the highest quality commercially available.

Enzyme and Antibody Preparations. Rats of Sprague-Dawley origin (200–250 g each; males unless noted otherwise) and male Swiss Webster mice (15–20 g each) were purchased from Harlan Industries (Indianapolis, IN). Male New Zealand white rabbits (2–3 kg each) were purchased from Hilltop Farms (Columbia, TN). Animals were treated with various compounds at the following dosages: PB, 80 mg kg^{-1} in 0.15 M NaCl administered ip once each day for 5 days; 3MC, 25 mg kg^{-1} in corn oil ip once each day for 3 days; BNF, 40 mg kg^{-1} in corn oil ip once each day for 3 days; TCDD, 5 μg kg^{-1} in 1,4-dioxane (20 μL) once ip 72 h prior to sacrifice; Aroclor 1254 , 300 mg kg^{-1} in corn oil once ip 72 h prior to sacrifice; DMN, 25 mg kg^{-1} in 0.15 M NaCl once ip 96 h prior to sacrifice; SO, 400 mg kg^{-1} in corn oil ip once each day for 5 days; PCN, 50 mg kg^{-1} in corn oil ip once each day for 3 days; and AAF, 0.05% (w/w) in feed for 6 days prior to sacrifice. Control animals received no chemicals. Animals were killed by a blow to the head and decapitation (rats), cervical dislocation (mice), or injection of 2 mL of 2 M KCl in the ear vein (rabbits). Tissue were removed and microsomes were prepared, with 0.4 mM phenylmethanesulfonyl fluoride in all buffers, as described elsewhere (Guengerich, 1977a). The preparation of human liver microsomes and lists of patient histories are presented elsewhere (Guengerich et al., 1981; Wang et al., 1980). Microsomes were stored prior to use at −70 °C in 10 mM Tris–acetate buffer (pH 7.4) containing 1 mM EDTA, 20% glycerol, and 0.4 mM phenylmethanesulfonyl fluoride. Protein was estimated as described by Lowry et al. (1951).

P-450s (B_2 fractions) were isolated from PB- and BNF-treated rats as described elsewhere (Guengerich & Martin, 1980; Guengerich, 1977b, 1978a). P-450 was prepared from PCN-treated rats with the procedure described by Elshourbagy & Guzelian (1980). Human liver P-450 was purified to apparent homogeneity from patients 6 and 18 as described elsewhere (Wang et al., 1980). P-450 LM-2 and P-450 LM-4 were isolated from PB- and BNF-treated rabbits, respectively, as described elsewhere (Coon et al., 1978; Haugen et al., 1975; Guengerich, 1977b). Antisera were raised and IgG fractions were prepared as described elsewhere (Kaminsky et al., 1981; Guengerich et al., 1981). When indicated, IgG fractions of antisera raised to rat liver P-450 BNF-B_2 were passed through columns of detergent-solubilized PB-treated rat liver microsomes coupled to agarose gel to prepare preadsorbed anti-P-450 BNF-B_2 (Thomas et al., 1979).

Electrophoresis and Staining Procedures. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out essentially as described by Laemmli (1970) with 0.15 × 14 × 10 cm slab gels unless indicated otherwise, with the exception that Pyronin Y was used as the tracking dye. Proteins were transferred from the polyacrylamide gels to nitrocellulose sheets with the general procedure of Towbin et al. (Towbin et al., 1979; Glass et al., 1981). The sheets were handled with disposable gloves and forceps, and cutting was done with a surgical scalpel in order to minimize background spotting not due to protein. A current setting of 200 mA was used for a period of 2 h for electrophoretic transfers. Nitrocellulose sheets, containing the transferred proteins, were shaken gently at 37 °C in a minimal amount of PBS containing 10% (v/v) calf serum and 3% (w/v) bovine serum albumin for 30 min to coat any reactive sites of the nitrocellulose. The sheets were washed 6 times with PBS (5 min each wash, with gentle shaking at room temperature and removal of buffer with an aspirator between washes). Each nitrocellulose sheet was then shaken with PBS containing 10% calf serum, 3% bovine serum albumin, and the appropriate antibody for 30 min at 37 °C and then for 12–16 h at 4 °C. In general, antisera were used at 1/100–1/200 dilutions or IgG fractions were diluted to 0.1 mg of protein mL^{-1}. Sheets were washed 6 times with PBS as before. Subsequently, each sheet was shaken with PBS containing 10% calf serum, 3% bovine serum albumin, and 2.5% (v/v) goat anti-rabbit IgG solution for 30 min at 23 °C. After six washes with PBS, the sheets were shaken with a 1/100 dilution of peroxidase–anti-peroxidase complex in PBS containing 10% calf serum and 3% bovine serum albumin for 30 min at 23 °C and then washed 6 times with PBS. To each tray containing a nitrocellulose sheet was added a fresh solution of 0.8 mM 3,3′-diaminobenzidine and 1.4 mM H_2O_2 in 50 mM Tris-HCl buffer (pH 7.6) (filtered through paper) to develop the stain. After 5 min of shaking at 23 °C, each sheet was washed 4 times with water. The visualized sheets were dried under filter papers and stored.

Photographs were taken over a fluorescent light box with Kodak Pan X-100 film. Densitometry was carried out with the nitrocellulose sheets by using a Corning 740 system or a

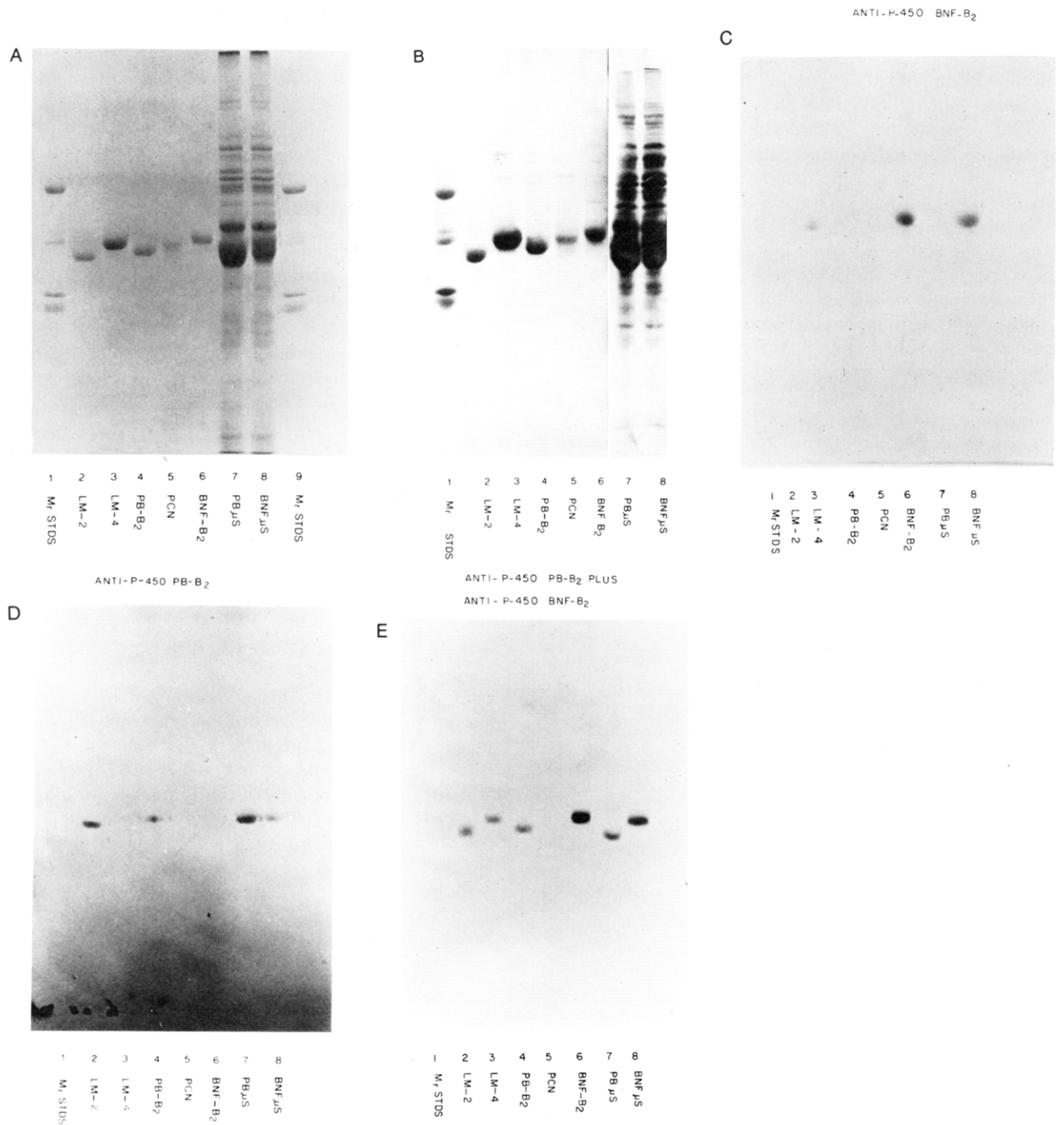

FIGURE 1: Electrophoresis of male rat liver microsomes and purified rat and rabbit liver P-450s. Electrophoresis was carried out in 0.4 × 0.15 × 10 cm lanes of a 7.5% (w/v) polyacrylamide gel according to Laemmli (1970). The anode was at the bottom of the gel. Individual samples electrophoresed in the various lanes included the following: 1 and 9, standard bovine serum albumin (accepted monomeric M_r 68 000), bovine liver catalase (M_r 58 000), *Escherichia coli* L-glutamate dehydrogenase (M_r 53 000), equine liver alcohol dehydrogenase (M_r 43 000), and rabbit muscle aldolase (M_r 40 000); 2, rabbit P-450 LM-2; 3, rabbit P-450 LM-4; 4, rat P-450 PB-B_2; 5, PCN-treated rat P-450; 6, rat P-450 BNF-B_2; 7, liver microsomes isolated from PB-treated rats; and 8, liver microsomes isolated from BNF-treated rats. In part A, 0.5 μg of each of the purified proteins or 10 μg of microsomal protein was electrophoresed in each well, and the gel was stained with Coomassie Brilliant Blue R-250 (Fairbanks et al., 1971). In part B, 5 μg of each of the purified proteins or 100 μg of microsomal protein was electrophoresed in each well. Proteins were transferred from the gel to a sheet of nitrocellulose, which was subsequently stained with Amido Black (Glass et al., 1981). In parts C–E, 0.15 μg of each of the purified proteins or 3 μg of microsomal protein was electrophoresed in each well. Proteins were transferred from the gel to a sheet of nitrocellulose, which was subequently treated with the described immunological staining procedure with anti-P-450 BNF-B_2 (part C), anti-P-450 PB-B_2 (part D), or a mixture of anti-P-450 PB-B_2 and anti-P-450 BNF-B_2 (part E).

Kontes Fiber-Optic instrument, utilizing fluorescent light in the reflectance mode. Areas were estimated either by triangulation or with the use of an automated integrator. Known amounts of purified P-450s were used on each polyacrylamide gel and nitrocellulose sheet to construct standard curves because of variation in staining intensity between different sheets.

Results and Discussion

Antibody Specificity. The major purified isozymes under consideration in this study were rat liver P-450 PB-B_2, rat liver P-450 BNF-B_2, rabbit liver P-450 LM-2, and rabbit liver P-450 LM-4. Electrophoretograms of the purified proteins are shown in Figure 1A with Coomassie Blue staining. The apparent monomeric molecular weights were 51 000, 55 000, 50 000, and 53 000, respectively. Also shown is purified PCN rat P-450 (M_r 53 500). The electrophoretogram in Figure 1B shows that all of these P-450s can be transferred to nitrocellulose. The patterns observed for the microsomes were

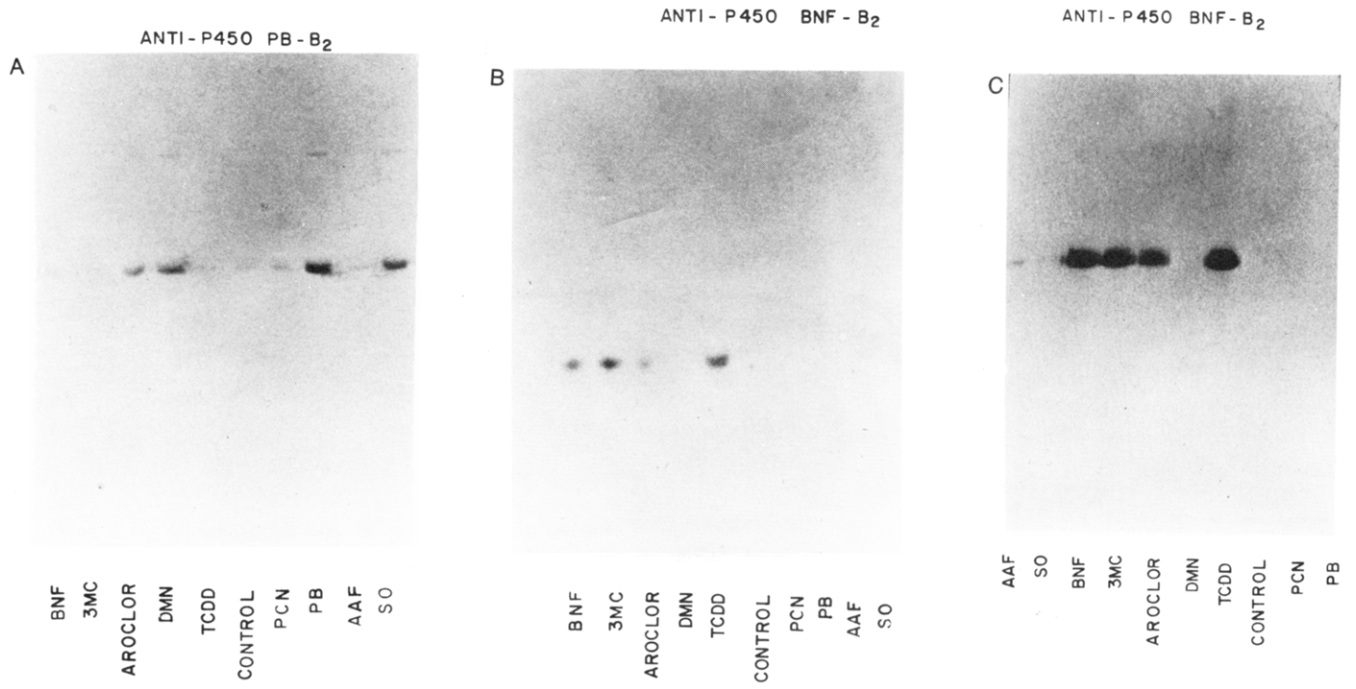

FIGURE 2: Immunoelectrophoresis of liver microsomes isolated from male rats treated with various compounds. Each well contained either 5 μg (part A) or 2 μg (parts B and C) of microsomal protein from rats treated as described with the indicated chemicals. The nitrocellulose sheet in part A was treated with anti-P-450 PB-B_2, the sheet in part B was treated with anti-P-450 BNF-B_2, and the sheet in part C was treated with anti-P-450 BNF-B_2 that had been preadsorbed with liver microsomes prepared from PB-treated rats. Rats were treated with the indicated inducers or were untreated (control).

similar to those obtained after staining of proteins within the gels (Figure 1A). When the residual acrylamide gel was stained with Coomassie Blue after electrophoretic transfer, the only microsomal protein detected was a small amount of high molecular weight material in the top 2 mm of the separating gel.

After proteins were transferred to nitrocellulose, staining using the described immunochemical procedure indicated a high degree of selectivity as well as sensitivity of the antibodies. For example, staining with anti-P-450 BNF-B_2 showed only purified P-450 BNF-B_2, rabbit P-450 LM-4, a protein migrating to the same position as P-450 BNF-B_2 in liver microsomes isolated from BNF-treated rats, and a small amount of the same protein in liver microsomes of PB-treated rats (Figure 1C). Some of the other anti-P-450 BNF-B_2 preparations also detected a faint band at M_r 58 000 in rat liver microsomes (regardless of pretreatment of the rats) that was not detected in purified P-450 BNF-B_2 preparations, and some of the anti-P-450 BNF-B_2 preparations did not react with rabbit P-450 LM-4 (vide infra). Staining with anti-P-450 PB-B_2 visualized rat P-450 PB-B_2, rabbit P-450 LM-2, a protein migrating to the same position as P-450 PB-B_2 in liver microsomes isolated from PB-treated rats, and a small amount of the same protein in liver microsomes of BNF-treated rats (Figure 1D). When the same sheets were stained with a mixture of the two antibodies, the differences in molecular weight values for the individual P-450s were readily observed (Figure 1E). Neither antibody recognized the P-450 purified from PCN-treated rats, in accord with the results of Elshourbagy & Guzelian (1980). Immunological cross-reactivity between rat P-450 PB-B_2 and rabbit P-450 LM-2 has been demonstrated previously by using an antibody raised to what would appear to be P-450 PB-B_2 or a similar protein (Kamataki et al., 1976; Thomas et al., 1976a). More recent work indicates that a monoclonal (hybridoma) antbody raised to rabbit P-450 LM-2 (Park et al., 1980) recognizes P-450 PB-B_2, and the two proteins form a pattern of fusion in double-diffusion immunoprecipitin analyses.[2] However, the point should be made that rat P-450 PB-B_2 and rabbit P-450 LM-2 are clearly different proteins as judged by molecular weight (Figure 1), peptide mapping (Guengerich, 1978b), and regioselectivity of metabolism (Kaminsky et al., 1980). While other work in this laboratory and others indicates that P-450 PB-B_2 and P-450 BNF-B_2 share some immunological similarity (Ryan et al., 1979; Guengerich, 1978a; Guengerich & Mason, 1979; Guengerich et al., 1981; Masuda-Mikawa et al., 1979), the cross-reactivity was not sufficient to be detected with this procedure. This observation may be the result of common antigenic sites associated with the heme moiety (Dus et al., 1980) or conformational properties that are lost during electrophoresis in the presence of sodium dodecyl sulfate. The sensitivity of the immunological staining technique is greater than that of staining with Coomassie Blue (Fairbanks et al., 1971) when the differences in the amounts of protein electrophoresed in Figure 1 are considered. No protein bands were visualized if preimmune sera were used at the same dilutions when either microsomes or purified proteins were electrophoresed and treated in this manner, and the differing results obtained with the specific antisera serve as further evidence of the lack of nonspecific staining. The basic results presented in Figure 1, as well as those presented in succeeding figures, were repeatable with a number of different antisera from different rabbits immunized with each form of P-450.

P-450 Induction in Rat Liver. The ability to identify individual forms of P-450 by molecular weight as well as immunological reactivity provided the opportunity to compare various agents known to induce P-450 in terms of the isozymes whose levels are changed. The data presented in Figure 2A indicate that PB, SO, DMN, and Aroclor 1254 all induce proteins of the same apparent molecular weight (51 000), corresponding to P-450 PB-B_2. On the other hand, a poly-

[2] S. S. Park, T. Fujino, D. West, F. P. Guengerich, and H. V. Gelboin, unpublished results.

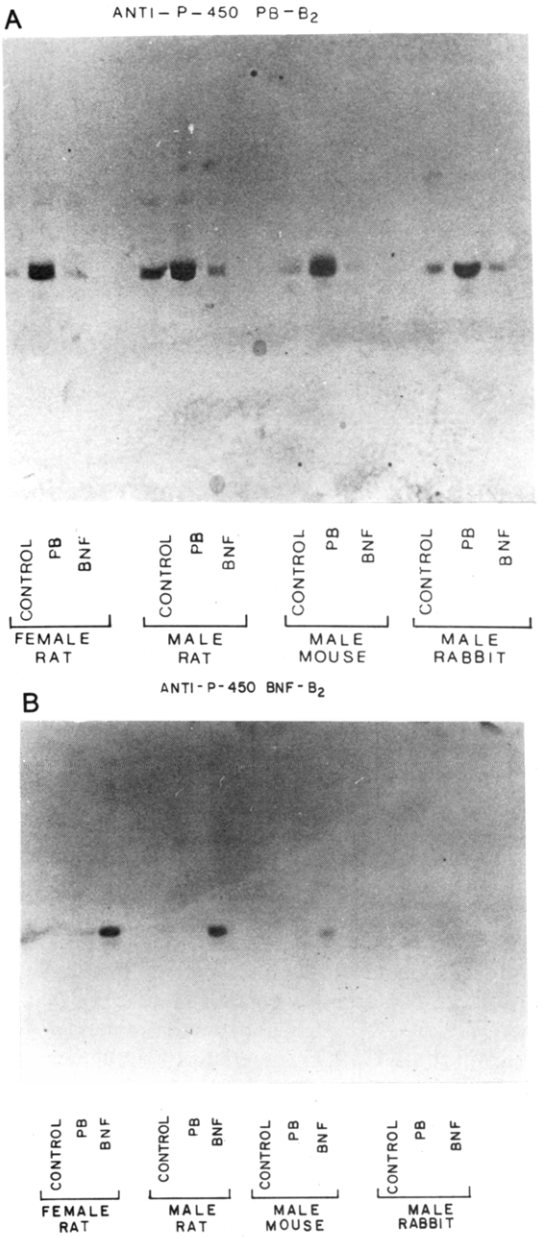

FIGURE 3: Immunoelectrophoresis of liver microsomes isolated from female rats, male rats, male mice, and male rabbits. The animals were either untreated (control) or treated with PB or BNF. Each well contained either 5 μg (part A) or 2 μg (part B) of microsomal protein. The nitrocellulose sheet in part A was treated with anti-P-450 PB-B_2, and the sheet in part B was treated with anti-P-450 BNF-B_2.

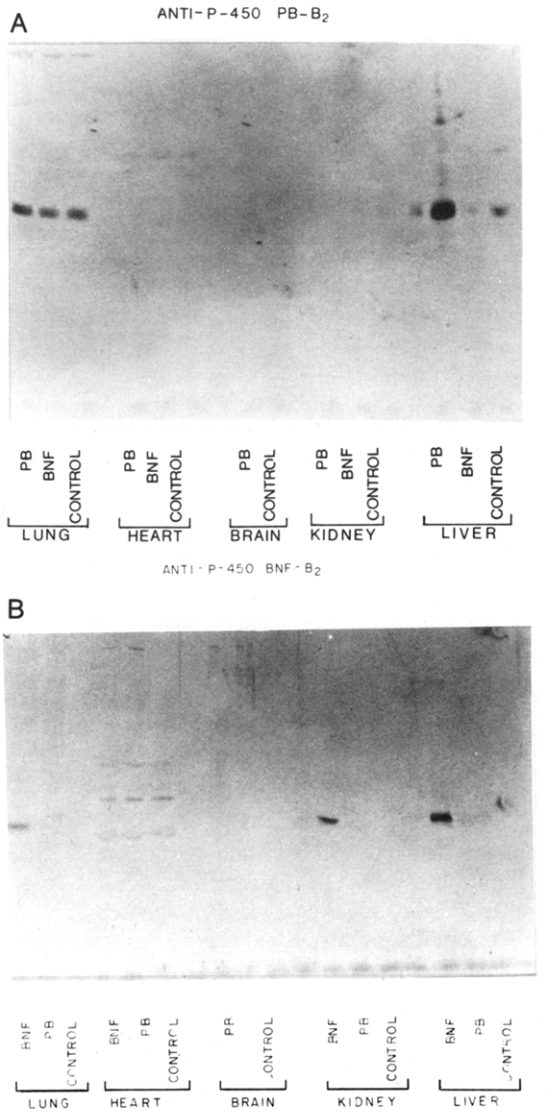

FIGURE 4: Immunoelectrophoresis of microsomes prepared from various tissues of male rats. The rats were either untreated (control) or treated with PB or BNF. Either 3 μg of liver microsomes or 30 μg of extrahepatic microsomes was used in each case. The nitrocellulose sheet in part A was treated with anti-P-450 PB-B_2, and the sheet in part B was treated with anti-P-450 BNF-B_2.

peptide with a higher molecular weight (55000) corresponding to P-450 BNF-B_2 is induced by BNF, 3MC, TCDD, and Aroclor 1254 (Figure 2B). Data similar to that reported in Figure 2B were obtained when anti-P-450 BNF-B_2 was preadsorbed with immobilized liver microsomes derived from PB-treated rats to remove less specific antibody populations (Figure 2C). Other studies support the view that the major P-450s induced by BNF and 3MC are identical as judged by immunological and catalytic activity (Guengerich & Martin, 1980; Guengerich et al., 1981; Kaminsky et al., 1980; Gozukara et al., 1981). The observation that the crude polychlorinated biphenyl mixture Aroclor 1254 induced both forms of P-450 is consistent with previous reports (Ryan et al., 1979; Alvares et al., 1973). Neither form of P-450 appeared to be induced by AAF or PCN.

Sex and Species Comparisons. Immunoelectrophoresis was also used to compare P-450s in male and female rats (Figure 3). Differences between the sexes were not dramatic, although the amount of the protein corresponding to P-450 PB-B_2 appeared to be somewhat lower in liver microsomes of untreated and BNF-treated females than in males (Figure 3A). Male rats, mice, and rabbits were also compared. With regard to the stains obtained with anti-P-450 PB-B_2, the mice appeared very similar to the rats with the exception that levels of the polypeptide were lower in untreated and BNF-treated mice than in rats. The protein recognized in rabbit liver microsomes with anti-P-450 PB-B_2 migrated further than P-450 PB-B_2, and other experiments demonstrated that the R_f was identical with that of P-450 LM-2 (see Figure 1). In all species examined in Figure 3A, a band was induced by PB, corresponding to P-450 PB-B_2 (rats, mice) or P-450 LM-2 (rabbits). Staining with anti-P-450 BNF-B_2 (Figure 3B) revealed a light band in BNF-treated mice corresponding to P-450 BNF-B_2, but no bands in BNF-treated rabbits were detected with this particular anti-P-450 BNF-B_2 preparation.

P-450 Induction in Extrahepatic Tissues. Extrahepatic rat tissue microsomes were also examined with immunoelectrophoresis (Figure 4). Anti-P-450 PB-B_2 did not visualize bands

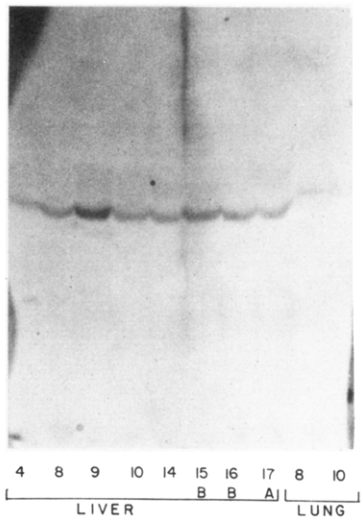

FIGURE 5: Immunoelectrophoresis of human liver and lung microsomes. Microsomes (400 μg of protein) were electrophoresed on a gel of 3-mm thickness. Patient identification is by number. The nitrocellulose sheet was treated with antibody raised to purified human liver microsomal P-450.

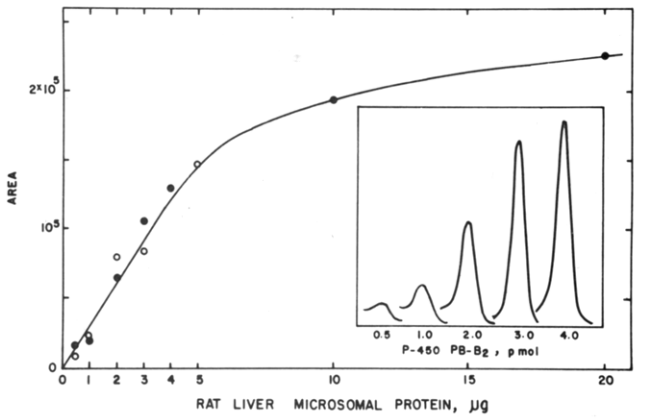

FIGURE 6: Linearity of densitometric response vs. amount of P-450. Varying amounts of liver microsomal protein derived from PB-treated rats were electrophoresed, transferred, stained, and quantitated by densitometry. The data are expressed in terms of relative area. Open and closed circles indicate data from two parallel experiments. The inset shows a typical series of peaks resulting from densitometry of varying levels of purified P-450 PB-B_2 treated in the same manner from a separate experiment.

in any of the kidney or brain samples analyzed, but a strong band with the same mobility as P-450 PB-B_2 was detected in lung microsomes regardless of the treatment of the rats (Figure 4A). Only a light band with an apparent molecular weight significantly greater than the antigen was found in all of the heart microsomes. Bands corresponding to P-450 BNF-B_2 were found only in microsomes of the liver, kidney, and lung of BNF-treated rats but not in any of the other samples (Figure 4B). These findings are in accord with the similarity in the patterns of regio- and stereoselective metabolism and anti-P-450 BNF-B_2 inhibition found with the substrate warfarin in the liver, kidney, and lung microsomes of 3MC-treated rats (Kaminsky et al., 1979). Double-diffusion immunoprecipitin studies with anti-P-450 3MC-B (similar to anti-P-450 BNF-B_2) showed a pattern of fusion formed between solubilized microsomes prepared from liver, kidney, and lung of 3MC-treated rats, and the precipitin lines formed by the reaction of anti-P-450 PB-B_2 with solubilized microsomes isolated from liver and lung of PB-treated rats also formed a pattern of fusion (Guengerich & Mason, 1979). Two bands were detected with anti-P-450 BNF-B_2 in all heart microsomes that had molecular weights above and below that of P-450 BNF-B_2 (Figure 4B).

Comparison of P-450s in Human Lung and Liver. An antiserum raised to purified human liver P-450 (patient 6) was also used to examine the existence of the same (or similar) forms of P-450 in various patients (Figure 5). All patients examined contained a detectable band with identical mobility (M_r 53 000) in liver microsomes. This band was not detected in lung microsomes derived from two of these patients, but a band with a higher molecular weight (57 000) was detected. This band is postulated to be a form of P-450 with similar immunological properties. As the band has a higher molecular weight, it is probably not an artifact of degradation. The data are consistent with a spur formed between liver and lung microsomes from patient 8 in double-diffusion immunoprecipitin analyses, indicative of partial immunological identity (Guengerich et al., 1981).

Quantitation of P-450s in Rat Tissues. The sensitivity of the immunological staining procedure, the discrimination between immunologically related proteins on the basis of apparent monomeric molecular weight, and the lack of need to radiolabel and repurify different antigens suggested that the procedure could be of use in quantitating individual forms of P-450. Stained bands could be scanned with a densitometer designed for thin layer chromatography plates and autoradiograms. Integration of the resulting peaks indicated that staining was directly proportional to the amount of microsomal protein or purified antigen applied to each well (Figure 6). With the saturating concentrations of the various antibodies used in these studies, the working level of detection was in the range of 0.5–5 pmol of P-450/well. Other studies using ^{125}I-labeled P-450 PB-B_2 indicated that the efficiency of transfer of the antigen from polyacrylamide gel to nitrocellulose was constant within this range of protein concentration (Towbin et al., 1979). The sensitivity of the method is approximately 1–2 orders of magnitude greater than methods such as radial immunodiffusion or rocket electrophoresis, which, in contrast, rely on precipitation of antigens by the primary antibody. While the method is not as sensitive as many competitive radioimmunoassay (Guengerich et al., 1981) and enzyme-linked immunesorbent assays, it does offer discrimination based on molecular weight and is easier to adapt to new antigen–antibody systems.

The method was used to estimate the levels of P-450 PB-B_2 and P-450 BNF-B_2 in hepatic and extrahepatic microsomes isolated from rats treated with various compounds (Table I). In all cases only the bands corresponding to the antigen in subunit molecular weight were used in making quantitative measurements. Since the antibodies appear to recognize cytochrome P-420 as well as P-450, the method of Johannesen & DePierre (1978) was used to estimate spectrally detectable levels of both P-450 and cytochrome P-420 for comparative purposes. The data presented in Table I support the general conclusions of Thomas et al. (1979, 1981) in that about two-thirds of the total P-450 present in liver microsomes of rats treated with PB or SO can be accounted for by the major PB-inducible form of P-450 (i.e., P-450 PB-B_2), that about three-fourths of the P-450 present in liver microsomes of rats treated with 3MC or BNF is attributable to the major BNF- (or 3MC-) inducible form (i.e., P-450 BNF-B_2), and that the levels of the two major inducible forms present in liver microsomes of Aroclor 1254 treated rats are similar in magnitude. Also, most of the P-450 in untreated or AAF- or PCN-treated rats is attributed to other forms. DMN appeared very similar to PB and SO in its effects on P-450 induction.

Induction of P-450 BNF-B_2 or a similar protein occurs in

Table I: Quantitation of Individual Forms of P-450 in Rat Tissue Microsomes[a]

inducer	specific content[b] total P-450 + cyt P-420[c]	specific content[b] P-450 PB-B_2[d]	P-450 PB-B_2/(total P-450 + cyt P-420) × 100	specific content[b] of P-450 BNF-B_2[d]	P-450 BNF-B_2/(total P-450 + cyt P-420) × 100
liver					
none	1.35	0.05	4	0.04	3
PB	3.79	2.58	68	0.04	1
AAF	0.70	0.01	2	0.03	4
SO	2.53	1.72	68	0.10	4
BNF	2.48	0.05	2	1.84	74
3MC	2.35	0.02	1	1.95	83
Aroclor 1254	4.31	1.63	38	1.85	43
DMN	3.39	2.67	79	0.13	4
TCDD	2.80	0.03	1	1.46	52
PCN	2.17	0.10	5	0.06	3
kidney					
none	0.45	<0.005	<1	0.005	<1
PB	0.46	<0.005	<1	0.005	<1
BNF	0.60	<0.005	<1	0.16	27
lung					
none	0.21	0.28	133	0.009	4
PB	0.19	0.31	164	0.019	10
BNF	0.24	0.30	125	0.33	140
heart					
none	1.00	<0.005	<1	0.005	<1
PB	1.27	<0.005	<1	0.005	<1
BNF	1.52	<0.005	<1	0.013	1
brain					
none	0.095	<0.005	<5	<0.005	<5
PB	0.14	<0.005	<3	<0.005	<3

[a] Electrophoresis, transfer, staining, and densitometry of P-450s were carried out with 0.5–30 μg of microsomal protein or 0.5–4 pmol of purified P-450 per well as described. Data are presented as means of two to eight determinations, and cytochrome P-420 is included with P-450 (Johannesen & DePierre, 1978) in making estimates of specific contents and percentages of individual isozymes. Variability of immunological specific content was on the order of ±10% of the values reported. [b] Nanomoles per milligram of protein. [c] Spectral specific content. [d] Immunological specific content.

kidney after BNF treatment, although not to the relative extent observed in liver. While warfarin hydroxylase activities are induced in rat kidney by PB (Kaminsky et al., 1979), the induced enzyme is insensitive to anti-P-450 PB-B_2 (Kaminsky et al., 1979), and the bulk of the P-450 in this tissue remains uncharacterized.

The induction pattern of P-450 BNF-B_2 or a similar protein in lung microsomes resembled that of liver, although discrepancy with spectral estimates was noted for this protein alone. The level of a protein closely related to P-450 PB-B_2 was greater than expected regardless of treatment. Perhaps the simplest explantation for the discrepancy is the existence of substantial amounts of apoenzyme in this tissue, unless the extinction coeffients used in the spectral measurements (Johannesen & DePierre, 1978) are grossly in error. Alternatively, a heteroclitic effect might be involved (Kabat, 1980). Nevertheless, the data suggest that a large portion of the P-450 in rat lung is immunologically very similar to P-450 PB-B_2. This situation resembles the case observed in rabbits, where a large portion of the uninduced lung P-450 is highly similar to the major liver P-450 induced by PB, i.e., P-450 LM-2 (Serabjit-Singh et al., 1979; Guengerich, 1977c). However, catalytic data on lung microsomes suggest that the activities may be too low to support the view that the major form of P-450 in untreated rat lung is identical with liver P-450 PB-B_2 (Kaminsky et al., 1979). The available data suggest that very little of the P-450 in rat heart or brain is highly similar to either P-450 PB-B_2 or P-450 BNF-B_2 (Table I).

Quantitation of P-450s in Human Liver. An antiserum raised to a purified preparation of human liver P-450 was also used to quantitate the levels of a P-450 with the same molecular weight, which reacted with the antibodies (Table II). The results indicate that the levels of this P-450 (or of closely related P-450s of the same molecular weight) varied from 0.08 to 0.42 nmol/mg of microsomal protein and that the portion of total human liver P-450 attributable to this form ranged from 6% to 56% (mean = 30%). Thus, the data suggest that the purified antigen used to raise the antisera represents a significant portion of human liver P-450. The average percentage of P-450 recognized by the antisera in this assay procedure is similar to the extent of inhibition of *d*-benzphetamine *N*-demethylase activity (but not benzo[*a*]pyrene

Table II: Quantitation of Human Liver P-450 Reacting with Antisera Raised to Purified Human Liver P-450[a]

patient no.	specific content[b] total P-450 + cyt P-420[c]	specific content[b] P-450 HL18[d]	P-450 HL18/(total P-450 + cyt P-420) × 100
4	0.80	0.25	31
8	0.58	0.25	43
9	0.68	0.22	32
10	0.47	0.16	34
14	0.64	0.10	16
15	0.75	0.42	56
16	0.69	0.29	42
17	0.60	0.12	20
18	0.83	0.15	18
19	1.38	0.80	6

[a] Electrophoresis, transfer, staining, and densitometry of P-450s were carried out with 10–20 μg of human liver microsomal protein or 0.5–4 pmol of purified P-450 (from patient 18, denoted P-450 HL18) per well as described. Data are presented as means of two to four determinations, and cytochrome P-420 is included with P-450 (Johannesen & DePierre, 1978) in making estimates of specific contents and percentages of total P-450. [b] Nanomoles per milligram of protein. [c] Spectral specific content. [d] Immunological specific content.

hydroxylase activity) observed with the antibodies (Guengerich et al., 1981).

Quantitation of P-450 in Rabbit Liver. In other studies, anti-P-450 PB-B_2 was used with purified rabbit P-450s LM-2 and LM-4 to quantitate levels of those proteins in rabbit liver microsomes. In untreated rabbits, 5% of the total liver P-450 was accounted for by P-450 LM-2 and 22% by P-450 LM-4. In BNF-treated rabbits, 2% of the total liver P-450 was attributed to P-450 LM-2 and 59% to P-450 LM-4. In PB-treated rabbits, 52% of the liver P-450 was attributed to P-450 LM-2 and 20% to LM-4. Thus, the system can be used for quantitation of a cross-reactive protein other than the homologous antigen if that purified protein is available.

Conclusions

We have used a recently developed immunoelectrophoretic technique to gain information about quantitative as well as qualitative aspects of P-450 multiplicity. Such information provides a basis for interpretation of quantitative immunohistochemical localization work in rat liver and extrahepatic tissues (Baron et al., 1981). The sensitivity of the method presented, its ability to discriminate among related proteins, and its adaptability to quantitative measurements are all viewed as useful in this field. These methods can be used effectively to determine the similarity of new inducing agents to those previously characterized. As more purified forms of P-450 and their antibodies become available, such techniques can be utilized in the estimation of the amounts of each form present in crude mixtures and the effects of various regimens upon these levels. The method has already been useful in examining specificity of antigen–antibody systems (Guengerich et al., 1981).[2] While sensitivity is usually not a matter of major concern with the major forms of rat liver P-450, studies with minor forms of hepatic P-450, with extrahepatic P-450s, and with human P-450s are aided by such sensitivity. The methodology not only is applicable to P-450s but also could find use with other proteins that exist in multiple forms and can be separated by any electrophoretic procedures. The method might also find use in comparing peptide maps (Guengerich, 1978b) and identifying common antigenic sites.

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