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Multiplicity, Strain Differences, and Topology of Phenobarbital-Induced Cytochromes P-450 in Rat Liver Microsomes†

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ABSTRACT: The multiplicity of phenobarbital-induced cytochromes P-450 in liver microsomes from male rats was investigated by using two-dimensional gel electrophoresis, peptide fingerprinting, and immunoaffinity chromatography. Two colonies each of Holtzman and Long-Evans rats were studied. Four molecular forms of phenobarbital-induced cytochromes P-450 were distinguished as polypeptides (designated PB3, variant PB3, PB4, and PB5) which showed apparent immunochemical identity and ≥95% fingerprint homology. Two of these polypeptides corresponded to cytochrome P-450b [Ryan, D., Thomas, P. E., Korzeniowski, D., & Levin, W. (1979) *J. Biol. Chem. 254*, 1365–1374] and cytochrome P-450e [Ryan, D., & Levin, W. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1640] which had been purified from Long-Evans rats (variant PB3 and PB5, respectively). Each rat colony was characterized by unique combinations of two or three of these immunochemically related forms of cytochrome P-450. Cytochrome P-450e was present in rats from all four colonies, but cytochrome P-450b was only found in Long-Evans rats. Polypeptide PB3 was only found in the two colonies of Holtzman rats, whereas polypeptide PB4 was present in one colony each of Holtzman and Long-Evans rats. In addition to these forms of cytochrome P-450, rats from each colony also evidenced three other major phenobarbital-induced polypeptides which gave unique fingerprints, and one of these was identified as representing epoxide hydrolase. Proteolytic digestion studies of intact microsomes demonstrated that the four immunochemically identical forms of cytochrome P-450 were partially exposed on the outer (cytoplasmic) surface of microsomes. However, polypeptide PB3 was characterized by the greatest rate of proteolytic degradation. These results clearly demonstrate that phenobarbital-induced cytochromes P-450 include microheterogeneous proteins which show remarkable variations related to rat strain and/or colony.

It is generally accepted that the ability of mammalian liver monooxygenase systems to metabolize a remarkably wide range of xenobiotic and endogenous substrates results from the participation of multiple forms of cytochrome P-450, each of which may exhibit a broad but selective substrate specificity (Lu & Levin, 1974). The number of unique cytochromes P-450 that potentially constitute the liver monooxygenase system in a single mammalian species is currently unknown. Nevertheless, at least six distinct forms of this enzyme have already been purified from rat liver (Guengerich, 1978; Ryan et al., 1979; Elshourbagy & Guzelian, 1980; Ryan et al., 1980; Ryan & Levin, 1981), and four of these have been shown to represent different gene products (Ryan et al., 1980; Botelho et al., 1979). However, in addition to their primary structures, it is also possible that cytochrome P-450 polypeptides differ as a result of posttranslational modifications [e.g., by glycosylation (Haugen & Coon, 1976; Hiwatashi & Ichikawa, 1980) and/or phosphorylation (Sharma et al., 1978)] which

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could further increase the molecular complexity and functionality of the system.

In an effort to evaluate the molecular diversity of cytochromes P-450 in rat liver microsomes, we modified a two-dimensional IF/NaDodSO$_4$[1] electrophoretic technique (O'Farrell, 1975) to resolve polypeptides that compose this fraction (Vlasuk & Walz, 1980). Liver smooth microsomes from male Holtzman rats were submitted to this analysis. The results indicated that the levels of several microsomal membrane polypeptides were significantly increased following phenobarbital treatment, and these were proposed to represent different forms of cytochrome P-450 (Vlasuk & Walz, 1980).

The work reported here extends our study of phenobarbital-induced polypeptides to include two colonies each of Holtzman and Long-Evans rats. Four closely related, phenobarbital-induced cytochromes P-450 were present in liver microsomes from these different groups of rats. These forms were distinguished in IF/NaDodSO$_4$ gels as resolved polypeptides (designated PB3, variant PB3, PB4, and PB5). However, they showed apparent immunochemical identity and evidenced ≥95% peptide fingerprint homology. Two of these forms were identified as cytochrome P-450b (variant PB3) (Ryan et al., 1979) and cytochrome P-450e (PB5) (Ryan & Levin, 1981) which have already been purified to homogeneity. Cytochrome P-450b, a major hemoprotein induced by phenobarbital in Long-Evans rats (Thomas et al., 1979, 1981), was only found in this strain, whereas cytochrome P-450e was present in rats from all four colonies. Furthermore, liver microsomes from each colony of phenobarbital-treated rats were characterized by unique combinations of two or three of these immunochemically identical polypeptides. Polypeptide PB3 was exclusively found in Holtzman rats and evidenced the greatest rate of proteolysis vis-ā-vis other immunochemically identical forms in digestions of intact microsomes. However, all of these closely related protein species in microsomes were susceptible to proteolysis, which indicated that they are exposed on the outer (cytoplasmic) surface of the microsomal membrane. These findings suggest the possibility that epigenetic factors may contribute to cytochrome P-450 multiplicity.

Experimental Procedures

Animals. Male rats were obtained from several sources, including a Holtzman colony from the Department of Psychology at Kent State University, the Charles River Breeding Laboratories, Inc., Wilmington, MA (Holtzman-derived and Long-Evans rats), and Blue Spruce Farms, Inc., Altamont, NY (Long-Evans rats). Both immature (3–4 weeks) and sexually mature (6–8 weeks) animals were used. The rats were maintained on a 12-h light/dark cycle in a well-ventilated animal room. Wire bottom stainless steel cages were used, and the animals were fed Purina rodent chow and water which were provided ad lib.

Phenobarbital Treatment and Microsome Preparation. Phenobarbital treatment consisted of daily intraperitoneal injections (75 mg/kg of body weight; in water) administered at the same time each day for 4 days. In most cases animals were starved for 24 h after the final (fourth) injection prior to killing by decapitation. Control animals were injected with an equivalent volume of water. Liver smooth microsomes (Vlasuk & Walz, 1980) or ribosome-stripped total microsomes (Lu & Levin, 1972) from groups of at least four rats were prepared as previously described. Total microsomal cytochrome P-450 was determined by the method of Omura & Sato (1964), and the protein was assayed according to Lowry et al. (1951) with bovine serum albumin as a standard.

Electrophoresis of Polypeptides from Microsomes and Microsomal Subfractions. Two-dimensional IF/NaDodSO$_4$ gel electrophoresis was performed as previously described (Vlasuk & Walz, 1980). Acrylamide concentrations in the second-dimensional gels were either 10%, 7.5%, or a gradient from 9% to 12%, which depended on the application. In lieu of Coomassie blue staining (Vlasuk & Walz, 1980), a silver staining technique (Oakley et al., 1980) was used for some IF/NaDodSO$_4$ gels. One-dimensional NaDodSO$_4$–polyacrylamide gel electrophoresis was accomplished by using 7.5% gels (Laemmli, 1970).

Polypeptide Fingerprinting. Coomassie blue stained polypeptides resolved in IF/NaDodSO$_4$ gels were excised and submitted to ^{125}I-labeled tryptic peptide fingerprinting according to the method of Elder et al. (1977) as modified by Zweig & Singer (1979). Approximately 5×10^5 cpm of ^{125}I-labeled peptides were used for electrophoresis/chromatography. In cofingerprints, half of this amount of radioactivity was applied for each of the two samples. Autoradiography was usually conducted for 6 days at −70 °C by using Kodak SB-5 film.

Immunological Procedures. Antibodies against cytochrome P-450b (Ryan et al., 1979) were raised in rabbits (Thomas et al., 1976) and made monospecific as previously described (Thomas et al., 1979). The monospecific IgG was specifically purified by immunoaffinity chromatography on a column of partially purified cytochrome P-450b covalently bound to Sepharose 4B. The specifically purified monospecific anti-cytochrome P-450b was then covalently bound to Sepharose 4B and used for the immunoaffinity purification of microsomal proteins (Thomas et al., 1979, 1981).

An immunoaffinity column (5.0 mL) was washed with 10 mL of 4 M potassium thiocyanate and equilibrated with 15 mL of 10 mM potassium phosphate buffer (pH 7.4) containing 0.2 M KCl, 20% glycerol, 0.1 mM EDTA, 0.2% Emulgen 911, and 0.5% sodium cholate. Each microsomal sample in ~2 mL of 0.25 M sucrose, containing 205–296 nmol of cytochrome P-450 (72–145 mg of protein), was solubilized by dilution to 10 mL with 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 0.5% sodium cholate, and 0.2% Emulgen 911. The solubilized microsomal sample was applied to the immunoaffinity column which was then washed with 3 column volumes of the column equilibration buffer, followed by 3 column volumes of 0.1 M borate buffer (pH 8.4) containing 1.0 M KCl and 0.2 mM EDTA, and finally with 3 column volumes of 0.2 M sodium acetate buffer (pH 4.2) containing 1.0 M KCl and 0.2 mM EDTA. The tightly bound cytochrome P-450 was subsequently eluted with 3 column volumes of 2 M potassium thiocyanate. This fraction was first dialyzed against 0.05 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1% sodium cholate and then concentrated by using an Amicon apparatus with a PM 30 membrane. The same immunoaffinity column was used for each experiment. Prior to each use, the column was washed with 2 M potassium thiocyanate before the column equilibration buffer.

Ouchterlony double-diffusion plates were prepared as previously described (Thomas et al., 1981).

[1] Abbreviations: IF/NaDodSO$_4$, two-dimensional polyacrylamide gel electrophoresis using isoelectric focusing in the first dimension followed by sodium dodecyl sulfate electrophoresis in the second dimension; NaDodSO$_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

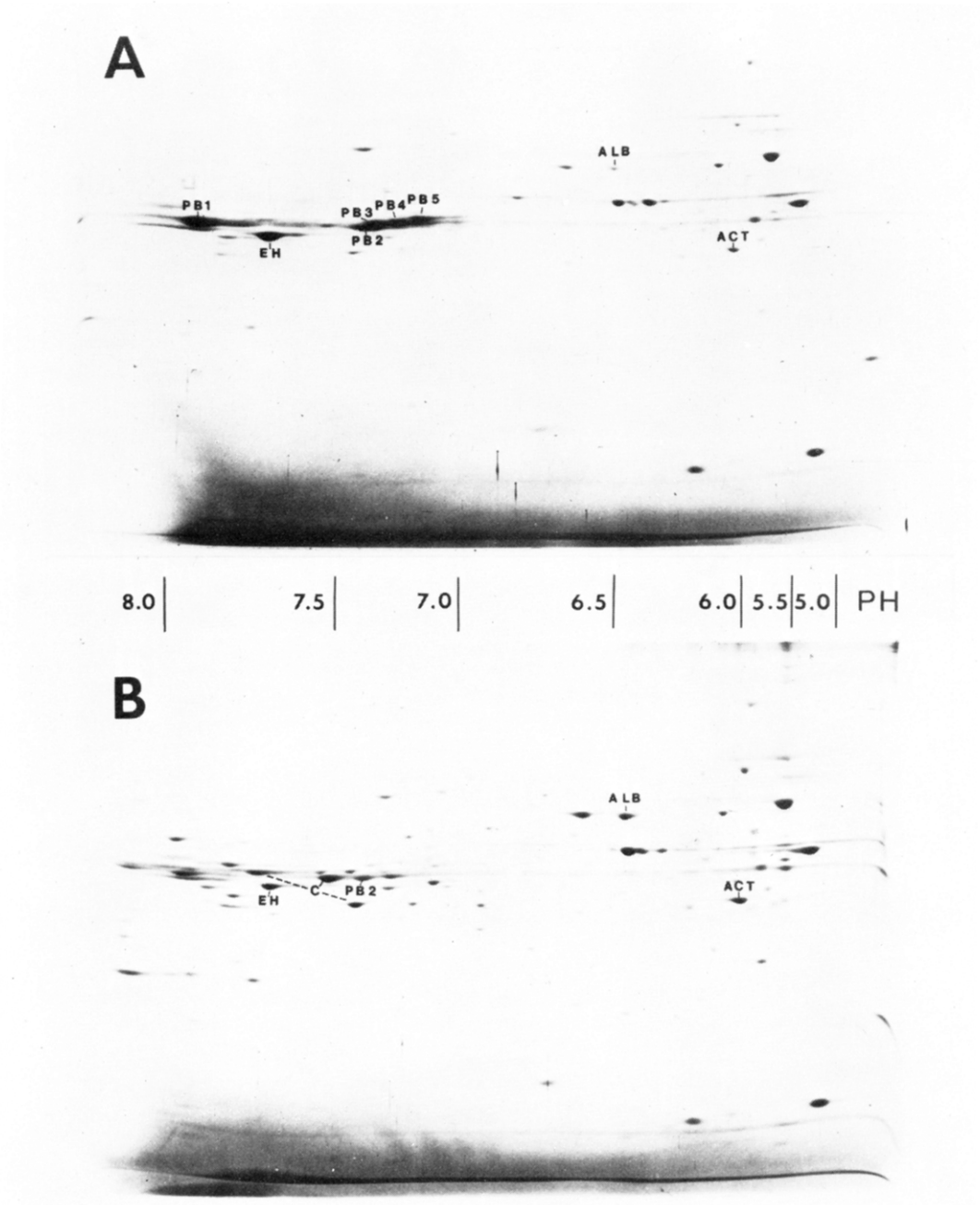

FIGURE 1: Two-dimensional IF/NaDodSO$_4$ gel electrophoretograms of liver smooth microsomes from phenobarbital-treated and untreated sexually mature, male Holtzman rats. (A) Microsomes from phenobarbital-induced animals (50 μg of protein); (B) microsomes from untreated animals (100 μg of protein). The approximate pH gradient for isoelectric focusing in the first dimension is illustrated on the horizontal axis. NaDodSO$_4$–polyacrylamide gel electrophoresis in the second dimension (9–12% acrylamide) was run from top to bottom on the vertical axis. The designations ALB and ACT refer to albumin (Vlasuk et al., 1980) and actin (unpublished observation) which serve as internal molecular weight standards of 67 000 and 43 000, respectively. Polypeptides PB1 through PB5 represent putative cytochromes P-450 that significantly increase as a result of phenobarbital treatment. Polypeptide EH, which also increases with phenobarbital treatment, has been identified as epoxide hydrolase by fingerprinting.[3] Polypeptides indicated by C may represent cytochromes P-450 in control microsomes that significantly *decrease* as a result of phenobarbital treatment. A lower amount of protein was applied for microsomes from phenobarbital-induced rats to enhance the resolution of polypeptides PB3–PB5. Coomassie blue staining was used; other details are found under Experimental Procedures.

Proteolytic Digestions of Microsomes. Digestions were accomplished in 0.25 M sucrose, 5 mM $MgCl_2$, 25 mM KCl, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.5, at 30 °C for 1 h (Cooper et al., 1980). Microsomal concentrations were 10 mg (protein)/mL, and protease concentrations were either 0.1 or 0.2 mg/mL. Microsomes that were used as controls for digestion were incubated under identical conditions in the absence of proteases. Incubations were terminated by diluting the reaction mixture with 2 volumes of "lysis buffer" which was used for sample solubilization prior to IF/NaDodSO$_4$ gel electrophoresis (Vlasuk & Walz, 1980).

Purification of Cytochromes P-450. Cytochrome P-450b (Ryan et al., 1979) and cytochrome P-450e (Ryan & Levin, 1981) were purified to apparent homogeneity from immature male Long-Evans rats as described.

Chemicals. Phenobarbital, Tris (Trizma base), α-chymotrypsin, and subtilisin BPN′ protease were obtained from Sigma Chemical Co. L-Tosylamido-2-phenyl chloromethyl ketone treated trypsin was a product of Worthington Biochemical Corp. All other chemicals were the best grade available.

Results and Discussion

Induction of Liver Microsomal Polypeptides by Phenobarbital Treatment of Holtzman Rats. Two-dimensional IF/NaDodSO$_4$ gels which illustrate phenobarbital-dependent

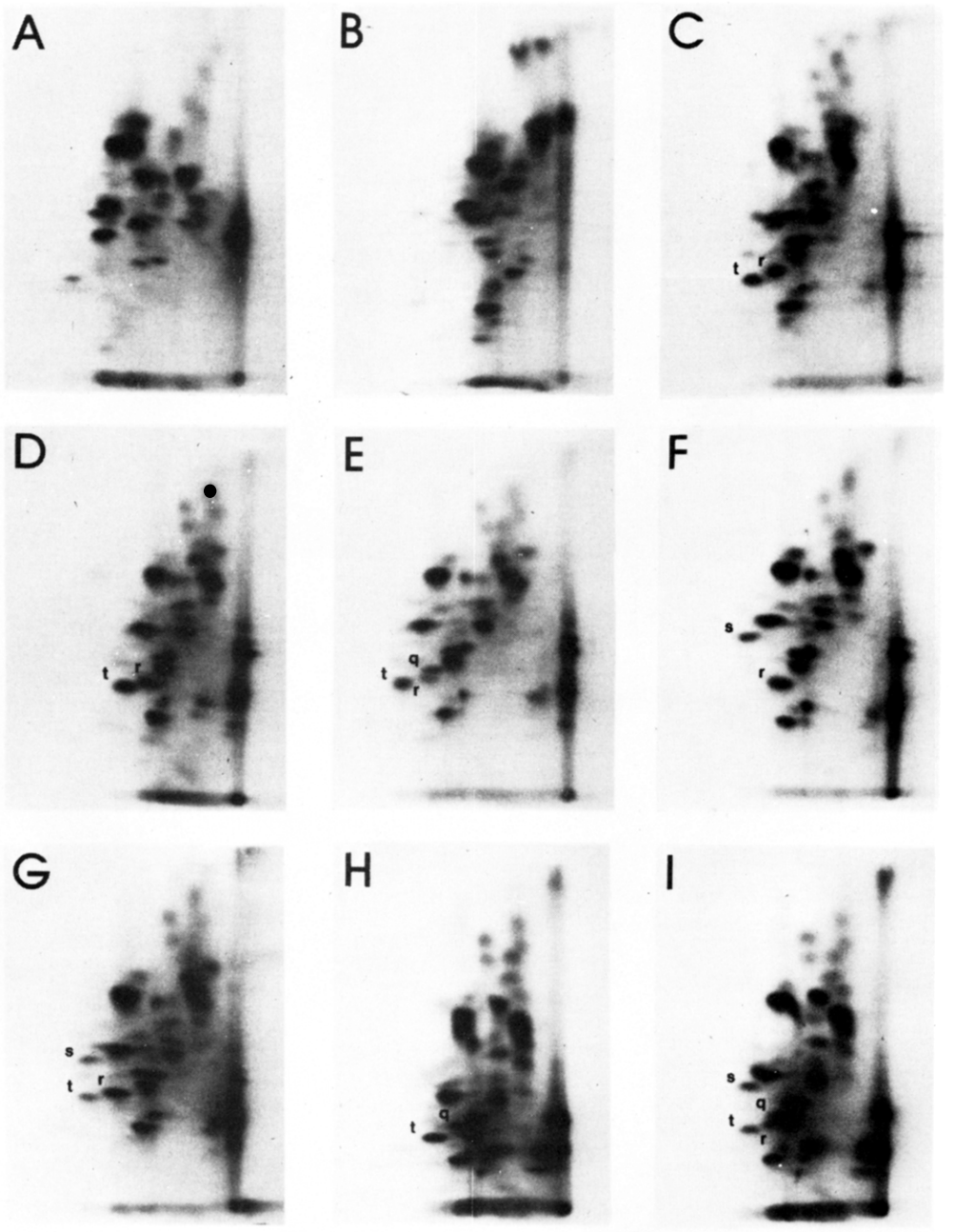

FIGURE 2: Radioiodinated peptide fingerprints of phenobarbital-induced polypeptides from Holtzman rat liver microsomes and purified cytochromes P-450. (A) Polypeptide PB1; (B) polypeptide PB2; (C) polypeptide PB3; (D) polypeptide PB4; (E) polypeptide PB5; (F) cytochrome P-450b; (G) cofingerprint of polypeptide PB3 and cytochrome P-450b; (H) cytochrome P-450e; (I) cofingerprint of cytochromes P-450b and P-450e. Polypeptides PB1 through PB5 and the purified cytochromes P-450 were excised from IF/NaDodSO$_4$ gels similar to those in Figure 1A and Figure 7B, respectively. Origins of the fingerprints are in the lower right corners. Electrophoresis was from right (anode) to left (cathode), and chromatography was from bottom to top. Peptides designated q, r, s, and t are characteristic for the different polypeptides (see text for details). Other conditions are found under Experimental Procedures.

changes in liver microsomal polypeptides for sexually mature, male Holtzman rats are shown in Figure 1. Similar results were observed in eight independent experiments, and the polypeptides designated PB1 through PB5 were consistently observed to substantially increase as a result of phenobarbital treatment.[2] These patterns of phenobarbital-induced polypeptides were independent of whether smooth microsomes (Vlasuk & Walz, 1980) or ribosome-stripped total microsomes (Lu & Levin, 1972) were used. In addition to their being significantly increased in response to phenobarbital treatment, these five polypeptides were also characterized by the following criteria which suggested that they represent cytochromes P-450: (a) they are major protein components of microsomes; (b) they are not released from microsomes after dilute deoxycholate treatment; (c) they evidence apparent molecular weights from ~51 000 to ~54 000; (d) they are basic proteins (Vlasuk & Walz, 1980). The polypeptide designated EH (Figure 1A), which is also induced by phenobarbital, has been identified as epoxide hydrolase by comparing its ^{125}I-labeled tryptic peptide fingerprint with that for the purified rat liver enzyme.[3] The results of this study will establish that poly-

[2] The results in Figure 1 differ slightly from those presented in a preliminary report (Vlasuk & Walz, 1980) as a consequence of improved polypeptide resolution in IF/NaDodSO$_4$ gel electrophoresis and by greater care in ensuring that the control rats were the same age.

[3] G. P. Vlasuk and F. G. Walz, Jr., unpublished experiments.

peptides PB3, PB4, and PB5 represent closely related forms of cytochrome P-450.

For characterization of the phenobarbital-induced polypeptides PB1-PB5 in Holtzman rat liver microsomes, they were excised from Coomassie blue stained IF/NaDodSO$_4$ gels and submitted to ^{125}I-labeled tryptic peptide fingerprinting (Elder et al., 1977; Zweig & Singer, 1979). Representative autoradiographs of the resolved tryptic peptides are shown in Figure 2. It was observed that polypeptides PB1 and PB2 gave unique fingerprints whereas those for PB3, PB4, and PB5 appeared to be identical, with the exception of a single difference in the fingerprint for PB5 (peptide labeled q in Figure 2E). Notwithstanding the consistent observation of this latter difference, a possible explanation of these extensive similarities is that polypeptides PB3, PB4, and PB5 represent the same molecular species artifactually resolved into three bands after isoelectric focusing in the first dimension of IF/NaDodSO$_4$ gel electrophoresis. This possibility has already been discussed (Vlasuk & Walz, 1980) and can be eliminated for the following reasons: (a) these polypeptides always migrate with slightly different mobilities in second-dimensional NaDodSO$_4$ electrophoresis (Figure 1A); (b) a purified protein representing polypeptide PB5 appears as a single species in IF/NaDodSO$_4$ gels; (c) the presence of some but not all of these polypeptides is dependent on the strain and/or colony of rats used; (d) the rates of proteolysis for these polypeptides are not the same in intact microsomes (evidence in support of points b–d is presented below).

Radioiodinated tryptic peptide fingerprints were also prepared for several purified forms of liver cytochrome P-450 [i.e., cytochromes P-450a, P-50b, P-450c (Ryan et al., 1979), P-450d (Ryan et al., 1980), and P-450e (Ryan & Levin, 1981)] which were isolated from male Long-Evans rats. The only homologies observed in comparisons of these fingerprints with those for phenobarbital-induced polypeptides PB1–PB5 in Holtzman rat liver microsomes involved cytochrome P-450b and cytochrome P-450e whose finterprints are shown in parts F and H of Figure 2, respectively. The lack of significant homologies with cytochromes P-450a, P-450c, and P-450d was not surprising since these proteins migrate to different coordinates in IF/NaDodSO$_4$ gels than those for polypeptides PB1-PB5.[3] The fingerprint for cytochrome P-450b appears to be identical in all respects with those for PB3 and PB4 with the exception of a single peptide (compare peptide t in parts C and D of Figure 2 for PB3 and PB4, respectively, with peptide s in Figure 2F for cytochrome P-450b). For verification of this apparent homology and single difference, a combination fingerprint was prepared by mixing ^{125}I-labeled tryptic peptides from polypeptide PB3 with the same amount of radioactivity as that for tryptic peptides from cytochrome P-450b, prior to electrophoresis/chromatography (Figure 2G). In addition to corroborating the extensive fingerprint homology of polypeptide PB3 with P-450b, this cofingerprint indicated that peptides s and t do not differ with respect to charged groups since they both show the same mobility in the electrophoretic dimension. These results suggest that cytochrome P-450b is not present in liver microsomes from phenobarbital-treated Holtzman rats, even though it is a major form of cytochrome P-450 in liver microsomes from phenobarbital-treated Long-Evans rats (Thomas et al., 1979; Thomas et al., 1981).

The fingerprint for purified cytochrome P-450e (Figure 2H) was identical in all respects with that for microsomal polypeptide PB5 (Figure 2E) with the exception of peptide r which characterizes the latter. However, peptide r also characterizes polypeptides PB3 and PB4 which are known to exhibit "tailing" toward the acidic end of IF/NaDodSO$_4$ gels (cf. Figure 3). Therefore, it is likely that the presence of peptide r in the fingerprint for PB5 is due to contamination of this polypeptide by PB3 and/or PB4. A further indication that cytochrome P-450e is identical with polypeptide PB5 is that the purified enzyme comigrates with this polypeptide in IF/NaDodSO$_4$ gel electrophoersis (see below). Finally, the obvious similarities between the fingerprints characterizing cytochrome P-450b and cytochrome P-450e were verified by their cofingerprint (Figure 2I) which highlights their minor differences (i.e., peptides r and s vis-à-vis peptides q and t, respectively).

Some apparent peptide differences between individual fingerprints were not considered significant since they were neither reproducible nor verified by cofingerprinting. It is important to note that the fingerprinting technique used here only reveals tryptic peptides which contain iodinatable tyrosine and/or histidine residues. Therefore, this method could fortuitously indicate a greater or lesser degree of amino acid sequence homology than really exists.

Induction of Specific Forms of Cytochrome P-450 by Phenobarbital Is Dependent on Rat Strain and/or Colony. The apparent absence of cytochrome P-450b per se in liver microsomes from phenobarbital-treated Holtzman rats indicated the need for further investigations of phenobarbital-induced polypeptides in Long-Evans and Holtzman rats. The Holtzman rats used in the experiments already described were from a colony maintained at Kent State University (referred to as Holtzman-KSU rats) which was continually replenished with new stock from the Holtzman Co. Holtzman-derived and Long-Evans rats were obtained from the Charles River Breeding Laboratories, Inc. (Holtzman-CR and Long-Evans-CR rats, respectively). Long-Evans rats from Blue Spruce Farms (Long-Evans-BSF rats) were also studied since they were the source for purifying cytochromes P-450b and P-450e (Ryan et al., 1979; Ryan & Levin, 1981). Liver microsomal polypeptide patterns in IF/NaDodSO$_4$ gels for *untreated* sexually mature males from these four groups were essentially the same as that illustrated in Figure 1B. In contrast, the patterns of phenobarbital-induced polypeptides were unique for each of these groups as shown in Figure 3A–D. For a given colony of animals, the same characteristic pattern was observed in completely independent experiments that were repeated from 2 to 8 times. Furthermore, the patterns of induced polypeptides PB1–PB5 for adult Holtzman-CR and Long-Evans-BSF male rats were identical with those for immature animals (i.e., 25-day-old males)[4] and, at least for Holtzman-CR rats, were independent of both the route (i.e., either drinking water or intraperitoneal injection) and the duration (i.e., 1–4 days) of phenobarbital administration (results not shown). These results suggest that the unique phenobarbital-induced polypeptide patterns in Figure 3A–D are characteristic of the various strains and/or colonies of rats tested.

Qualitative differences in phenobarbital-induced polypeptides among the different groups of rats only involved those species that isoelectrically focused to the same coordinates as those for Holtzman-KSU polypeptides PB3 and PB4 (cf. Figure 1A). These coordinates and that for PB5 are illustrated in Figure 3 for reference. Direct comparison of the IF/NaDodSO$_4$ patterns (i.e., by overlaying stained gels on a light

[4] Age-related differences in putative cytochrome P-450 polypeptides which are not induced by phenobarbital have been observed in untreated male rats (unpublished observations).

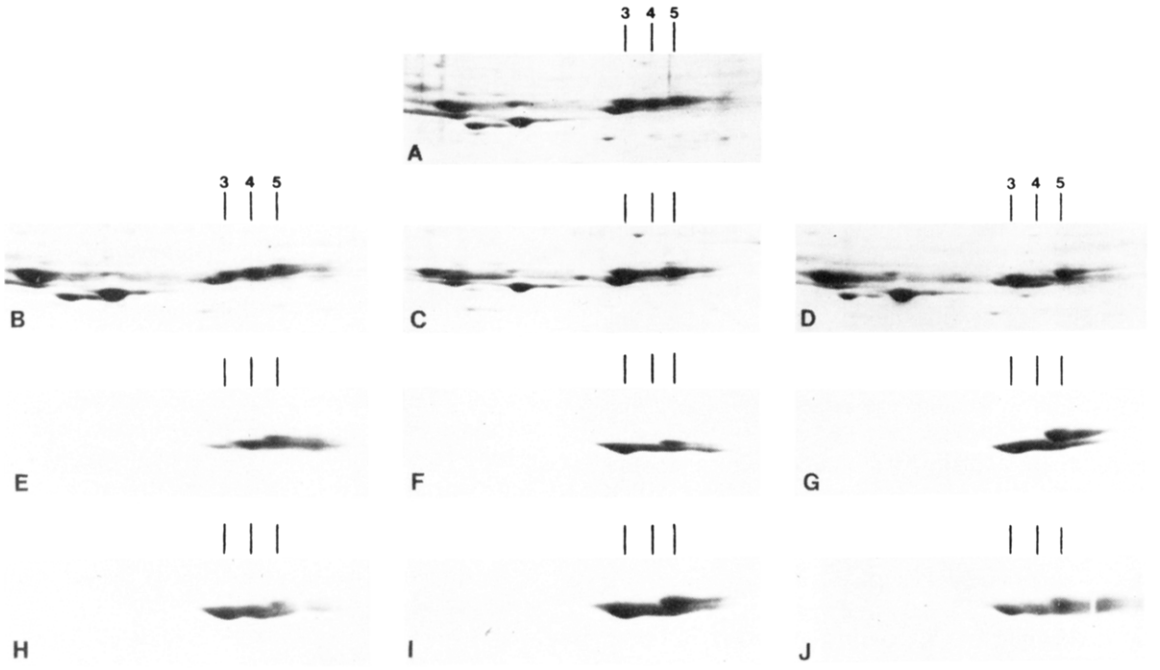

FIGURE 3: Portions of two-dimensional IF/NaDodSO$_4$ gels for liver microsomes from different colonies of phenobarbital-treated Holtzman and Long-Evans rats and corresponding microsomal immunoaffinity purified fractions isolated by using specifically purified anti-cytochrome P-450b immunoaffinity chromatography. Equivalent portions of IF/NaDodSO$_4$ gels are presented, and their locales can be estimated by referring to Figure 1. All microsomes and immunoaffinity purified fractions were from phenobarbital-treated adult male rats. (A) Long-Evans-CR microsomes (50 μg of protein); (B) Holtzman-KSU microsomes (50 μg of protein); (C) Long-Evans-BSF microsomes (50 μg of protein); (D) Holtzman-CR microsomes (50 μg of protein); (E) immunoaffinity purified fraction from Holtzman-KSU microsomes (4 μg of protein); (F) immunoaffinity purified fraction from Long-Evans-BSF microsomes (4 μg of protein); (G) immunoaffinity purified fraction from Holtzman-CR microsomes (4 μg of protein); (H) combined immunoaffinity purified fractions from Holtzman-KSU and Long-Evans-BSF microsomes (4 μg of protein each); (I) combined immunoaffinity purified fractions from Holtzman-CR and Long-Evans-BSF microsomes (4 μg of protein each); (J) combined immunoaffinity purified fractions from Holtzman-KSU and Holtzman-CR microsomes (4 μg of protein each). IF/NaDodSO$_4$ gel electrophoresis of microsomes employed 9–12% gradient gels in the second dimension and Coomassie blue staining. IF/NaDodSO$_4$ gel electrophoresis of immunoaffinity purified fractions employed 7.5% gels (0.75 mm thick) in the second dimension and silver staining. The designations 3, 4, and 5 refer to the first-dimensional isoelectric focusing coordinates for polypeptides PB3, PB4, and PB5 in Holtzman-KSU microsomes. Further details are found under Experimental Procedures.

box) suggested that Holtzman-KSU polypeptide PB5 is found in every microsomal preparation tested, whereas polypeptide PB4 is present in significant quantities only in Holtzman-KSU and Long-Evans-CR microsomes. Furthermore, Holtzman-KSU polypeptide PB3 is present in microsomes from Holtzman-CR rats, whereas a *variant* polypeptide PB3 appears in microsomes from Long-Evans-CR and Long-Evans-BSF rats. This variant polypeptide migrates to the same isoelectric focusing coordinate as polypeptide PB3 but evidences a slightly lower mobility in the second dimension of IF/NaDodSO$_4$ gel electrophoresis (compare parts A and C with parts B and C of Figure 3). Therefore, it appears that four variable, closely related phenobarbital-induced polypeptides exist in the different groups of rats studied: polypeptide PB3 (present in both colonies of Holtzman rats), variant PB3 (present in both colonies of Long-Evans rats), polypeptide PB4 (present in Holtzman-KSU and Long-Evans-CR rats), and polypeptide PB5 (i.e., cytochrome P-450e; present in all four rat groups).

All nine of the polypeptides indicated in Figure 3A,C,D were fingerprinted. Polypeptides PB3 from Holtzman-CR rats and PB5 from Holtzman-CR, Long-Evans-BSF, and Long-Evans-CR rats had essentially the same fingerprints as their counterparts from Holtzman-KSU rats (cf. Figure 2C,E, respectively). Figure 4A–C illustrates fingerprints for the three closely related, phenobarbital-induced polypeptides resolved from Long-Evans-CR rat liver microsomes. The fingerprint for variant polypeptide PB3 (Figure 4A), which is characterized by peptide s, is identical with that for purified cytochrome P-450b (Figure 2F), as confirmed by cofingerprinting (data not shown). This result was also obtained for variant polypeptide PB3 from Long-Evans-BSF rat liver microsomes. The fingerprint for Long-Evans-CR polypeptide PB5 (Figure 4C) is virtually the same as that for Holtzman-KSU polypeptide PB5 (Figure 2E), being characterized by peptide. As already discussed, PB5 has the same fingerprint as purified cytochrome P-450e from Long-Evans-BSF rats. Finally, the fingerprint for Long-Evans-CR polypeptide PB4 (Figure 4B) is identical with that for its corresponding Holtzman-KSU polypeptide (Figure 2D). This latter identification, which was corroborated by a cofingerprinting experiment (Figure 4D), is interesting since variant polypeptide PB3 (i.e., cytochrome P-450b) and polypeptide PB4 have the same mobility in the second dimension of NaDodSO$_4$ gel electrophoresis (see Figure 3A). Therefore, in addition to having distinct coordinates in IF/NaDodSO$_4$ gels, two of the four closely related polypeptides (variant PB3 and PB5) found in the different colonies of rats are characterized by unique fingerprints, whereas polypeptides PB3 and PB4 appear to have identical fingerprints. The distinct arrays of these closely related polypeptides in liver microsomes form the four colonies of rats examined are summarized in Table I. Even though these qualitative differences in phenobarbital-induced cytochromes P-450 exist among the different groups of rats tested, it is interesting that the specific content of total cytochrome P-450 was essentially the same (i.e., 3.2 $\pm$ 0.3 nmol of cytochrome P-450/mg of protein for smooth microsomes).

Evidence has been presented which indicates that differences in liver cytochromes P-450 exist between rat strains (Dent et al., 1980; Guengerich et al., 1981). However, the molecular basis for these differences has not yet been elucidated. The

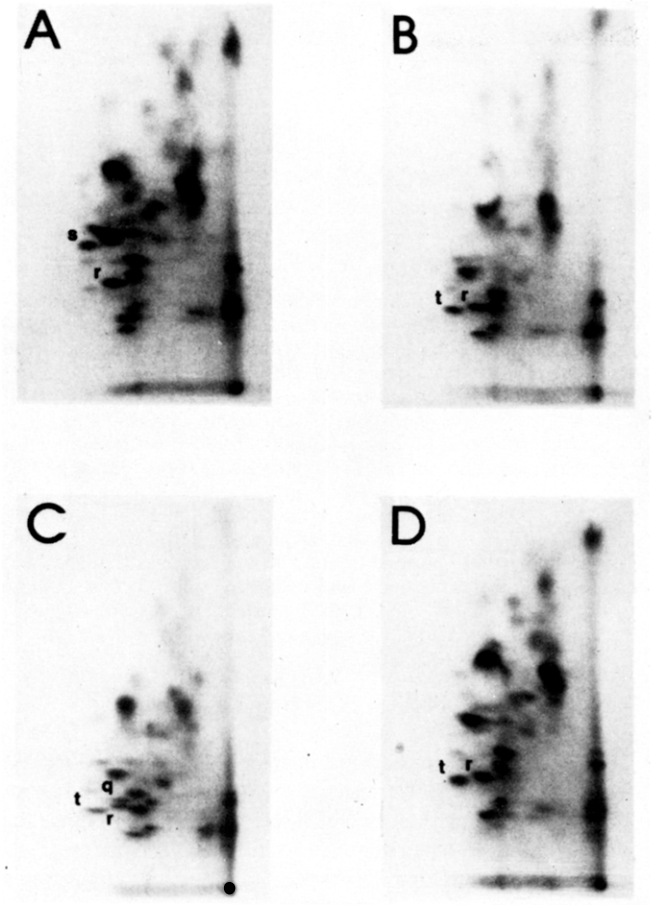

FIGURE 4: Radioiodinated peptide fingerprints of phenobarbital-induced polypeptides from Long-Evans-CR rat liver microsomes. (A) *Variant* polypeptide PB3; (B) polypeptide PB4; (C) polypeptide PB5; (D) cofingerprint of polypeptide PB4 from Long-Evans-CR with that from Holtzman-KSU microsomes. Variant polypeptide PB3 has the same coordinate in first-dimensional isoelectric focusing as polypeptide PB3 but was characterized by a lower mobility in the second dimension of IF/NaDodSO$_4$ gel electrophoresis (see Figure 3A). Designations and conditions were the same as those in Figure 2.

Table I: Distribution of Closely Related, Phenobarbital-Induced Liver Microsomal Polypeptides in Different Rat Strains and Colonies[a]

	polypeptides[c]			
rat strain colony[b]	variant PB3 (P-450b)	PB3	PB4	PB5 (P-450e)
Long-Evans-BSF	++	–	–	+
Long-Evans-CR	++	–	+	+
Holtzman-KSU	–	++[d]	++[d]	+
Holtzman-CR	–	++	–	+

[a] The identifications of specific polypeptides as resolved species in IF/NaDodSO$_4$ gels were as described in the text. [b] Nomenclature is described in the text. [c] P-450b, cytochrome P-450b; P-450e, cytochrome P-450e; PB3, PB4, and PB5 as designated in Figure 1A and Figure 3A–D. Relative amounts estimated from Coomassie blue stained gels (see text); (+) present, (–) absent, and (++) present in greatest relative amount. [d] The relative amount of polypeptides PB3 and PB4 varied in eight separate experiments; e.g., see Figure 1A and Figure 3B.

results of the present study suggest that discrete molecular forms of phenobarbital-induced cytochromes P-450 in rat liver microsomes not only represent differences among strains but also are dependent on the particular colony of animals investigated.

Immunochemical Relatedness of Four Forms of Phenobarbital-Inducible Cytochrome P-450. The extensive fin-

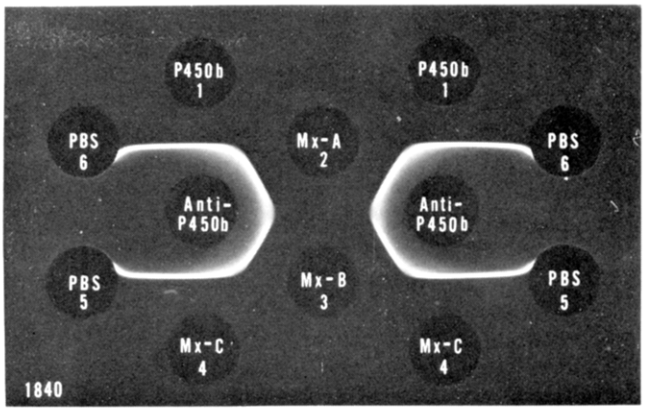

FIGURE 5: Ouchterlony double-diffusion analysis of solubilized microsomes from Holtzman and Long-Evans rats using antibody to cytochrome P-450b. The two central wells were filled with monospecific antibody to cytochrome P-450b [25 mg/mL (Thomas et al., 1981)]. The two wells marked 1 contained cytochrome P-450b (2.5 μM), well 2 contained solubilized microsomes from phenobarbital-treated Long-Evans-BSF rats (Mx-A), well 3 contained solubilized microsomes from phenobarbital-treated Holtzman-CR rats (Mx-B), and the two wells marked 4 contained solubilized microsomes from phenobarbital-treated Holtzman-KSU rats (Mx-C), all at 7 μM total cytochrome P-450. The wells marked 5 and 6 contained phosphate-buffered saline (PBS). Other conditions were as described under Experimental Procedures.

gerprint homologies among polypeptides PB3, variant PB3 (cytochrome P-450b), PB4, and PB5 (cytochrome P-450e) suggested that they could share antigenic determinants. So that this possibility could be tested, antibody made monospecific to cytochrome P-450b (Thomas et al., 1979) was used for Ouchterlony double-diffusion analysis of solubilized liver microsomes from phenobarbital-treated Long-Evans-BSF, Holtzman-CR, and Holtzman-KSU rats. This antibody preparation reacts with each of the microsomal samples to give a single precipitin band, and these form a line of identity with that for cytochrome P-450b (Figure 5). The absence of spurs at the intersections of these bands suggests that the antigenic component(s) in each microsomal sample is (are) immunochemically identical. These results were not unexpected since cytochrome P-450e, which is present in all of these microsome samples, has been shown to be immunochemically identical with cytochrome P-450b (Ryan & Levin, 1981).

For identification of the particular microsomal polypeptide(s) which are immunochemically related to cytochrome P-450b, the immunoreactive component(s) of solubilized liver microsomes from three groups of phenobarbital-treated rats were isolated by using immunoaffinity chromatography on a column containing specifically purified antibody to cytochrome P–450b. The three immunoaffinity purified fractions were subjected to IF/NaDodSO$_4$ analysis, and portions of the resulting gels (which contained >96% of the protein staining material resolved from these fractions) are shown in Figure 3E–G. Even though these gels were overloaded,[5] it is clear that the immunochemically related polypeptides reveal idiosyncratic patterns for each animal group which are congruent with those for corresponding polypeptides resolved from the original microsomes (compare Figure 3B–D with Figure 3E–G, respectively). Therefore, it appears that the immunoaffinity purified fraction for a given group of rats is char-

[5] Overloading of these polypeptides was revealed by their "tailing" toward the acidic end in the first dimension (isoelectric focusing) of IF/NaDodSO$_4$ gel electrophoresis. This phenomenon was verified by fingerprinting material in the "tails" and by observing IF/NaDodSO$_4$ gels which were overloaded with purified cytochrome P-450b.

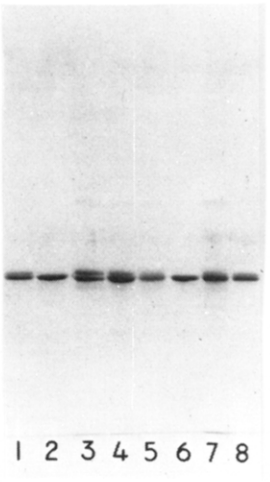

FIGURE 6: $NaDodSO_4$–polyacrylamide gel electrophoresis of cytochromes P-450b and P-450e and anti-cytochrome P-450b immunoaffinity purified fractions from phenobarbital-treated Holtzman-KSU, Holtzman-CR, and Long-Evans-BSF rats. (Lane 1) Cytochromes P-450b plus P-450e; (lane 2) cytochrome P-450b; (lane 3) immunoaffinity purified fraction from Holtzman-CR microsomes; (lane 4) immunoaffinity purified fraction from Holtzman-CR microsomes plus cytochrome P-450b; (lane 5) immunoaffinity purified fraction from Holtzman-KSU microsomes; (lane 6) cytochrome P-450b; (lane 7) immunoaffinity purified fraction from Long-Evans-BSF microsomes; (lane 8) cytochromes P-450b plus P-450e. Immunoaffinity purified fractions were the same as those in Figure 3. Acrylamide gels (7.5%) (0.75 mm thick) and Coomassie blue staining were used. Other conditions are described under Experimental Procecdures.

acterized by the same constituency of closely related polypeptides as found in the original microsomes (cf. Table I). This conclusion was verified by coelectrophoresis of immunoaffinity purified fractions and microsomes in $IF/NaDodSO_4$ gels and by fingerprinting of the resolved immunorelated polypeptides (results not shown).

For corroboration of evidence that the polypeptides listed in Table I are common to microsomes from the different groups of phenobarbital-treated rats and are characterized by unique electrophoretic coordinates, all binary combinations of the three different immunoaffinity purified fractions were coelectrophoresed in $IF/NaDodSO_4$ gels (Figure 3H–J). Examination of these results revealed that polypeptide PB3 is common to both groups of Holtzman rats and that polypeptide PB5 (cytochrome P-450e) is present in all three immunoaffinity purified fractions. In addition, the difference in second-dimensional mobility of polypeptides PB3 and variant PB3 (cytochrome P-450b) was confirmed (e.g., compare parts E and F with part H of Figure 3). A clearer demonstration of this latter difference was provided by $NaDodSO_4$–polyacrylamide gel electrophoresis which permitted direct comparisons of purified cytochromes P-450b and P-450e with the various immunoaffinity purified fractions (Figure 6). . Even though the resolution of individual immunorelated polypeptides was limited in this one-dimensional electrophoretic system, two distinct bands were observed for the immunoaffinity purified fraction from Holtzman-CR rats (Figure 6, lane 3), and these consist of polypeptides PB5 (cytochrome P-450e) and PB3 (top and bottom bands, respectively). Cytochrome P-450b migrated with slightly lower mobility than polypeptide PB3 (compare lanes 2 and 4 with lane 3) but with the same mobility as polypeptide PB4 which is the major immunorelated component from Holtzman-KSU microsomes (compare lanes 5 and 6). This latter observation illustrates the shortcoming of using one-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis as a definitive criterion to identify a specific form of

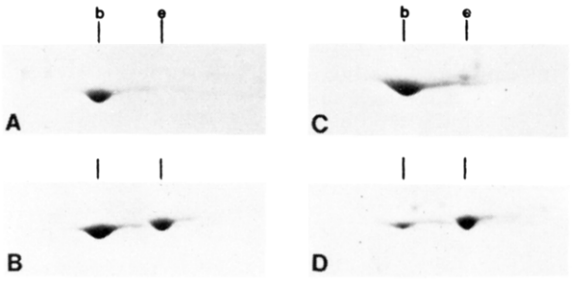

FIGURE 7: Portions of two-dimensional $IF/NaDodSO_4$ gels for cytochrome P-450b and P-450e in the absence and presence of the anti-cytochrome P-450b immunoaffinity purified fraction from phenobarbital-treated Long-Evans-BSF rats. The portions of $IF/NaDodSO_4$ gels presented are similar to those in Figure 3. (A) Cytochrome P-450b (1 μg); (B) cytochrome P-450b (1 μg) plus cytochrome P-450e (0.8 μg); (C) cytochrome P-450b (1 μg) plus Long-Evans-BSF immunoaffinity purified fraction (0.6 μg); (D) cytochrome P-450e (0.8 μg) plus Long-Evans-BSF immunoaffinity purified fraction (0.6 μg). Gels in the second dimension were 7.5% acrylamide (0.75 mm thick), and staining was with Coomassie blue. The immunoaffinity purified fraction was the same as that shown in Figure 3F. The designation b and e refer to the isoelectric focusing coordinates for cytochromes P-450b and P-450e, respectively. See Experimental Procedures for additional details.

cytochrome P-450. This problem has already been addressed in regard to cytochromes P-450b and P-450d (Ryan et al., 1980).

When purified cytochrome P-450b was submitted to $IF/NaDodSO_4$ analysis, it appeared as a single polypeptide spot (Figure 7A) at coordinates which were distinct from those for cytochrome P-450e, as demonstrated by coelectrophoresis of both pure samples (Figure 7B). This latter result demonstrates the advantage of $IF/NaDodSO_4$ gel electrophoresis over $NaDodSO_4$–polyacrylamide gel electrophoresis in resolving these cytochromes P-450 (compare Figure 7B with Figure 6, lanes 1 and 8). Variant polypeptide PB3 and polypeptide PB5 in Long-Evans rat liver microsomes were identified as representing cytochrome P-450b and cytochrome P-450e, respectively, on the basis of their fingerprints and their coordinates after resolution in separate $IF/NaDodSO_4$ gels (see above). These identifications were more concretely established by independently coelectrophoresing cytochrome P-450b and cytochrome P-450e with the immunoaffinity purified fraction from Long-Evans-BSF microsomes, which supposedly contained only these polypeptides (parts C and D of Figure 7, respectively).

The present work demonstrates that antibodies prepared against cytochrome P-450b were not strictly monospecific (Thomas et al., 1979, 1981) since they reacted equivalently with cytochrome P-450e and polypeptides PB3 and PB4. In view of this finding, the results of previous cytochrome P-450b immunoquantitation studies (Thomas et al., 1981) should be revised to include these other forms if they were present. In the case of microsomes from phenobarbital-induced, sexually mature, male Long-Evans-BSF rats, the cytochrome P-450 content measured with this antibody preparation ($\sim$57% of the total cytochrome P-450, Thomas et al., 1981) would include only cytochrome P-450e in addition to cytochrome P-450b since polypeptides PB3 and PB4 were not significantly present in these microsomes. In any event, cytochromes P-450b and P-450e plus trace amounts of cytochromes P-450a and P-450c account for only 60% of the total cytochrome P-450 content in these microsomes. How much of the remaining, unidentified cytochrome P-450 content (i.e., 40% of the total) represents induced or noninduced forms is currently unknown. There is circumstantial evidence that polypeptides PB1 and PB2 represent phenobarbital-induced cytochromes

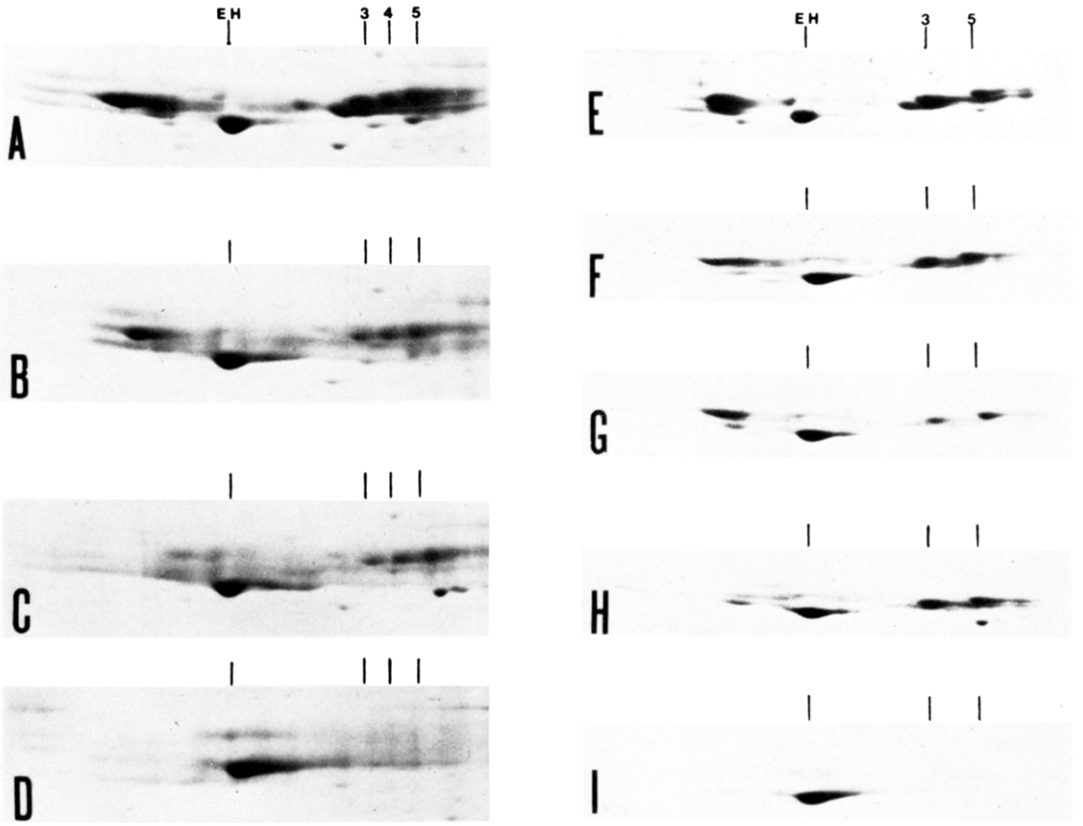

FIGURE 8: Portions of IF/NaDodSO$_4$ gels for microsomal polypeptides from phenobarbital-treated Holtzman rats before and after digestion with several proteases. (A and E) Undigested microsomes from phenobarbital-treated Holtzman-KSU (HKSU) and Holtzman-CR (HCR) rats, respectively; (B and F) chymotrypsin (0.1 mg/mL) treated microsomes from HKSU and HCR rats, respectively; (G) chymotrypsin (0.2 mg/mL) treated microsomes from HCR rats; (C and H) subtilisin (0.1 mg/mL) treated HKSU and HCR microsomes, respectively; (D and I) trypsin (0.1 mg/mL) treated HKSU and HCR microsomes, respectively. All digestions were conducted at 30 °C by using 10 mg/mL microsomal protein. Second-dimensional gels were 10% acrylamide for HKSU microsomes (A–D) and a gradient from 9% to 12% acrylamide for HCR microsomes (E–I). Each gel resolved 130 μg of microsomal protein and was stained with Coomassie blue. The designations EH and 3–5 are described in the legends to Figure 1 and Figure 3, respectively. Other details are presented under Experimental Procedures.

P-450 (see above). In addition, several major polypeptides in microsomes from control animals, which could represent other forms of cytochrome P-450, are considerably decreased as a result of phenobarbital treatment (see polypeptides designated C in Figure 1B). Therefore, it is possible that polypeptides PB1 and PB2 represent the bulk of unidentified cytochromes P-450 in microsomes from phenobarbital-treated animals.

Proteolytic Digestion of Phenobarbital-Induced Polypeptides in Intact Microsomes. The exposure of phenobarbital-induced cytochromes P-450 on the outer (Cytoplasmic) surface of microsomes has recently been evidenced in proteolytic digestion studies using spectrophotometric measurements of these hemoproteins (Cooper et al., 1980) and by specific antibody inhibition of microsomal cytochrome P-450 catalytic activity (Thomas et al., 1977). For examination of the transverse topology of *individual* cytochromes P-450 in the microsomal membrane, smooth microsomes from phenobarbital-treated Holtzman rats were digested with several proteases, and the polypeptides were subjected to IF/NaDodSO$_4$ gel electrophoresis (Figure 8). The microsomal membranes in the present study were judged to have remained intact during the course of digestion on the basis of the lack of digestion of albumin trapped in the microsomal lumen (Vlasuk et al., 1980). It was observed that phenobarbital-induced polypeptides PB1–PB5 in Holtzman rat liver microsomes were completely digested with trypsin after a 1-h incubation (compare parts A and E with parts D and I of Figure 8, respectively), which indicates that they are all quantitatively exposed on the outer (cytoplasmic) surface of microsomes. The same result was observed with microsomes from phenobarbital treated Long-Evans rats (data not shown). Digestions with chymotrypsin and subtilisin under similar conditions showed that the apparent *rate* of proteolysis for polypeptide PB3 was greater than that for polypeptides PB4 and PB5 (cytochrome P-450e) (Figure 8). These observations were confirmed in experiments conducted for various digestion times (15–60 min). In addition, 5-min digestions with trypsin also revealed the same phenomenon (results not shown). In order to correctly interpret these results, it is important that the size of the stained polypeptide spots in IF/NaDodSO$_4$ gels reflects the amount of resolved protein. This relationship has already been described (O'Farrell, 1975), and it has been our experience that the amount of ^{125}I incorporated into individual polypeptide spots excised from IF/NaDodSO$_4$ gels [i.e., as used for fingerprinting (Elder et al., 1977; Zweig & Singer, 1979)] is proportional to spot size. The results in Figure 8G show that the size of the remaining spot for polypeptide PB3 is actually smaller than that for polypeptide PB5 (cytochrome P-450e) after chymotryptic digestion, even though it was considerably larger in the untreated control (Figure 8E). Therefore, it may be concluded that the rate of degradation of polypeptide PB3 in microsomes is greater than that for polypeptides PB4 and PB5 (cytochrome P-450e).

These results provide an additional criterion that distinguishes polypeptide PB3 from the other molecular forms of immunochemically identical cytochromes P-450. The reason why polypeptide PB3 is more susceptible to proteolysis than polypeptides PB4 and PB5 in microsomes is currently unknown. However, it is reasonable to suggest that either its

intramolecular structure per se is less stable toward proteolysis or that it is exposed to a greater extent on the microsomal surface. A selective protection against proteolysis by the inducing drug does not seem likely since the same results were obtained when digestions were conducted in the presence of 0.1% phenobarbital (results not shown).

In summary, the closely related phenobarbital-induced cytochromes P-450 from the four rat colonies tested are composed of extremely similar polypeptides (PB3, variant PB3, PB4, and PB5) since they appear to be immunochemically identical and are characterized by peptide fingerprints that are ≥95% homologous. Nevertheless, evidence that this group of enzymes includes four distinct forms can be summarized as follows: (a) all forms migrate to unique coordinates in IF/NaDodSO$_4$ gels which remain the same regardless of whether the polypeptides are resolved from microsomes, immunochemically isolated fractions, or as individual purified proteins; (b) cytochromes P-450b and P-450e (variant PB3 and PB5, respectively) are chromatographically distinct on DEAE-cellulose columns and have been purified to apparent homogeneity (Ryan et al., 1979; Ryan & Levin, 1981); (c) three distinct peptide fingerprints characterize these four forms; (d) polypeptides PB3 and PB4 which yield indistinguishable fingerprints evidence different rates of proteolysis in intact microsomes; (e) the presence of these four forms varies depending on the strain and colony of rat used. Therefore, for the groups of rats studied, it may be concluded that phenobarbital induces four molecularly distinct forms of immunochemically related cytochromes P-450, and these include two newly identified species. Furthermore, one of these closely related forms (cytochrome P-450b) is only found in Long-Evans rats whereas the form corresponding to polypeptide PB3 is only found in Holtzman rats. Finally, two or three of these closely related cytochromes P-450 compose four unique arrays which distinctly characterize each of the four colonies of rats examined.

At this time it is not possible to judge whether these closely related molecular species of cytochrome P-450 represent genetically distinct polypeptides per se or result from post-translational modification(s) of one or several gene products. However, the subtle variations of these closely related forms of the enzyme in different rat colonies demonstrate that the population of cytochromes P-450 is subject to a much finer degree of control than previously shown.

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