Species Differences in Cytochromes P-450 and Epoxide Hydrolase: Comparisons of Xenobiotic-Induced Hepatic Microsomal Polypeptides in Hamsters and Rats†

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ABSTRACT: Cytochromes P-450 and epoxide hydrolase in hamsters were studied by using two-dimensional gel electrophoresis of hepatic microsomes from untreated animals and those treated with phenobarbital, 3-methylcholanthrene, β-naphthoflavone, *trans*-stilbene oxide, and pregnenolone-16α-carbonitrile. Coelectrophoresis with corresponding microsomes from rats and in situ peptide mapping were used to identify resolved microsomal polypeptides as cytochromes P-450 or epoxide hydrolase. Two forms of hepatic microsomal epoxide hydrolase were shown to exist in hamsters; these evidenced extensive structural homology with the corresponding enzyme in rats and were induced by the same xenobiotics. At least eight inducible polypeptides in microsomes from hamsters were tentatively identified as cytochromes P-450. Two of these were electrophoretically identical and structurally related with previously characterized forms of the enzyme in rats. Homologues of several major cytochromes P-450 induced by pregnenolone-16α-carbonitrile and/or phenobarbital in the rat were apparently not present in the hamster. In most cases, putative forms of inducible cytochrome P-450 in the hamster existed at significant levels in microsomes from untreated animals whereas in rats the levels of most inducible forms of the enzyme were low in control microsomes, being more strictly dependent on xenobiotic pretreatment. In contrast with epoxide hydrolase, the molecular complexity of hepatic cytochrome P-450 seems to be comparable for rats and hamsters, but the structure and control of these hemoproteins appear to have markedly diverged.

Hepatic cytochromes P-450 are hemoproteins which act as terminal monooxygenases in an endomembrane-bound system which metabolizes a broad variety of xenobiotics and endogenous substrates. At least eight distinct forms of hepatic microsomal cytochromes P-450 have been isolated from rats (Ryan et al., 1979, 1980, 1982a; Elshourbagy & Guzelian, 1980; Cheng & Schenkman, 1982; Waxman et al., 1982), and a comparable number have been purified from rabbits (Haugen et al., 1975; Koop et al., 1982). In most cases, structural and immunochemical analyses indicated that these enzymes represent unique gene products.

It has been known for some time that the aggregate level of hepatic cytochromes P-450 can be dramatically increased by treating animals with a variety of xenobiotics (Conney, 1967). Recent studies of hepatic microsomes from rats using immunochemical (Thomas et al., 1981) and high-resolution electrophoretic (Vlasuk et al., 1982b) methods to evaluate the levels of six cytochrome P-450 isozymes have shown that specific induction programs are enacted which depend on the particular xenobiotic treatment used. Furthermore, it appeared that repression of constitutive cytochromes P-450 may also result from xenobiotic treatment of rats (Vlasuk & Walz, 1982; Vlasuk et al., 1982b).

A comprehensive knowledge of cytochrome P-450 ensembles and their regulation by xenobiotics in different animal species is a prerequisite for a detailed understanding of their molecular evolution and teleology. Hepatic cytochromes P-450 have been most intensively studied in rats and rabbits; however, the total molecular complexity of these hemoproteins in either of these species is currently unknown. In addition, the structural relatedness of cytochromes P-450 from different animal species is poorly understood, even though some interesting amino acid sequence homologies have been shown to exist between purified rat and rabbit forms of the enzyme (Botelho et al., 1979; Fujii-Kuriyama et al., 1982) and similarities of some cytochromes P-450 from rats with those from humans (Wang et al., 1980) and mice (Chen et al., 1982) have been reported. Finally, species comparisons of induced hepatic microsomal proteins have employed one-dimensional sodium dodecyl sulfate ($NaDodSO_4$)[1] polyacrylamide gel electrophoresis analyses (Thorgeirsson et al., 1979) which have only a limited capacity for resolving the polypeptide constituents of microsomes (Vlasuk & Walz, 1980).

The present study attempts to use the information available from studies on the hepatic cytochrome P-450 system in rats as a means to elucidate some general aspects of the same system in a moderately related species of animal. In this work hepatic microsomal polypeptides from hamsters and rats treated with phenobarbital, 3-methylcholanthrene, β-naphthoflavone, *trans*-stilene oxide, and pregnenolone-16α-carbonitrile were directly compared by using two-dimensional IF/$NaDodSO_4$ electrophoresis (Vlasuk & Walz, 1980). Hamsters were chosen for comparison since they are inexpensive, well-characterized laboratory animals which are from the same order (Myomorpha) as rats but represent a different family (Cricetidae). It was also of interest to study hamsters since it has already been shown that a higher specific content of several hepatic microsomal monooxygenase activities characterize this species vis-à-vis rats (Thorgeirsson et al., 1979; Walker, 1978).

Two-dimensional IF/$NaDodSO_4$ gel electrophoresis of rat-hepatic microsomes is capable of resolving epoxide hydrolase and all known forms of cytochromes P-450 (Vlasuk

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; IF/$NaDodSO_4$ gel electrophoresis, two-dimensional polyacrylamide gel electrophoresis with isoelectric focusing in the first dimension and $NaDodSO_4$/polyacrylamide gel electrophoresis in the second dimension.

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et al., 1982b) as well as several allozymic strain variants of cytochromes P-450b and P-450e (Vlasuk et al., 1982a; Walz et al., 1982). Coelectrophoresis of microsomes and in situ peptide mapping were used in the present study to elucidate possible structural homologies between rat and hamster microsomal polypeptides. Evidence is presented which suggests the existence of two forms of hepatic microsomal epoxide hydrolase in hamsters. In general, the results indicate that the hepatic monooxygenase systems in rats and hamsters are clearly distinguished in terms of their cytochrome P-450 constituents and the regulation of these hemoproteins in response to different xenobiotic inducers.

Materials and Methods

Corn oil was obtained from Matheson Coleman & Bell, Inc. Phenobarbital was purchased from Sigma Chemical Co., and 3-methylcholanthrene was a product of Eastman Organic Chemicals, Inc. *trans*-Stilbene oxide and β-naphthoflavone were obtained from Aldrich Chemical Co. Pregnenolone-16α-carbonitrile was a gift from Monsanto Chemical Co. Deionized water having a specific resistance of at least 1 MΩ was used in all experiments, and other chemicals were reagent grade or the best grade available.

Hamsters [Lak: LVG(SYR)] were 50–55-day-old males obtained from Charles River Breeding Laboratories, Inc. Male Holtzman rats (50 days old; 180 ± 10 g) were obtained from a breeding colony at Kent State University. Groups of five hamsters or two rats were treated on 4 consecutive days with the following doses administered by intraperitoneal injection: phenobarbital in water, 75 mg/kg; 3-methylcholanthrene in corn oil, 25 mg/kg; pregnenolone-16α-carbonitrile in corn oil, 25 mg/kg; β-naphthoflavone in corn oil, 80 mg/kg; *trans*-stilbene oxide in corn oil, 400 mg/kg. Control animals were injected with either water or corn oil. Animals were starved for the final 24 h prior to killing by decapitation. Total hepatic microsomes were prepared as described previously (Lu & Levin, 1972). Protein concentrations were determined according to the method of Lowery et al. (1951) with bovine serum albumin as the standard. Cytochromes P-450 and P-420 in microsomes were analyzed according to Omura & Sato (1967).

Microsomal samples containing 80 μg of protein were submitted to two-dimensional IF/NaDodSO$_4$ gel electrophoresis which was performed as previously described (Vlasuk & Walz, 1980) by using a gradient of 9–12% acrylamide in the second dimension. Resolved polypeptides were stained with Coomassie blue, and gels were destained as reported before (Vlasuk & Walz, 1980). In experiments where microsomes were coelectrophoresed, samples containing 40 μg of hepatic microsomal protein from both rats and hamsters were used. In situ ^{125}I-labeled peptide mapping of Coomassie blue stained polypeptides in two-dimensional IF/NaDodSO$_4$ gels was performed as previously described (Vlasuk et al., 1982a).

Results

The specific contents of spectrophotometrically determined cytochromes P-450 and P-420 in hepatic microsomes from control rats and hamsters and those treated with phenobarbital, 3-methylcholanthrene, β-naphthoflavone, *trans*-stilbene oxide, and pregnenolone-16α-carbonitrile are presented in Table I. Phenobarbital, 3-methylcholanthrene, and β-naphthoflavone were effective agents for inducing cytochrome P-450 in both rats and hamsters. *trans*-Stilbene oxide and pregnenolone-16α-carbonitrile induced significant amounts of cytochromes P-450 in rats, whereas in hamsters *trans*-stilbene oxide caused only a marginal increase in the microsomal level of these hemoproteins and pregnenolone-16α-carbonitrile appeared to be an ineffective inducing agent.

Table I: Total Cytochromes P-450 and P-420 in Hepatic Microsomes from Xenobiotic-Treated Hamsters and Rats

	cytochromes P-450 + P-420 content[a]			
	rat		hamster	
treatment	nmol/mg	relative[b]	nmol/mg	relative[b]
control	0.901	100	1.40	100
phenobarbital	3.01	334	3.06	219
3-methylcholanthrene	2.22	246	3.27	234
β-naphthoflavone	1.95	216	2.76	197
trans-stilbene oxide	1.49	165	1.90	137
pregnenolone-16α-carbonitrile	1.80	200	1.61	115

[a] Cytochrome P-450 was greater than 95% of the total cytochromes P-450 plus cytochromes P-420 content in all cases. [b] As percent of control.

Table II: Major Polypeptides in Hepatic Microsomes from Rats and Hamsters Treated with Various Xenobiotics

polypeptide[a]		
rat	hamster	identification in the rat[b]
A	a	plasma protein precursor
B	b	NADPH-cytochrome P-450 reductase
C	c	unidentified
D	d	unidentified
E	e	proalbumin[c]
F	f	albumin[c]
G	g	unidentified
H	h	unidentified
I	i	cytochrome P-450d[d,i]
J	j	cytochrome P-450 PB-1[e]
K	k_1, k_2	epoxide hydrolase[d]
L	l	actin
M	m	NADH-cytochrome b_5 reductase
N	n	cytochrome b_5
O		putative cytochrome P-450 (polypeptide MCI)[f]
P	u	cytochrome P-450c[d,i]
Q		cytochrome P-450a[d]
R		cytochrome P-450 PCN[d]
S		putative cytochrome P-450 (polypeptide M2)[g]
T		immunochemically identical cytochromes P-450[h]

[a] Hamster and rat polypeptides are listed together on the basis of their similar migration in two-dimensional electrophoretograms (Figures 1 and 2). Additional hamster polypeptides which are tentatively identified as cytochromes P-450 are listed in Table III. [b] The identifications which are not referenced employed immunochemical, peptide fingerprinting, coelectrophoretic, or microsomal localization (topological) techniques. [c] See Vlasuk et al. (1980). [d] See Vlasuk et al. (1982b). [e] See footnote 4. [f] See Vlasuk & Walz (1982, 1980). [g] See Vlasuk & Walz (1982). [h] See Vlasuk et al. (1982a) and Walz et al. (1982). [i] Cytochromes P-450c and P-450d appear as streaks after IF/NaDodSO$_4$ electrophoresis (Vlasuk et al., 1982a).

The complete patterns of hepatic microsomal polypeptides for β-naphthoflavone-treated rats and hamsters are compared in Figure 1. All identified and some major unidentified polypeptides are labeled in these two-dimensional electrophoretograms, and these are listed in Table II. A more accurate species comparison of these hepatic microsomal polypeptides was obtained after their coelectrophoresis (Figure 2). The relative coordinates of various polypeptides in Figure 1 were used to distinguish rat and hamster polypeptides in Figure 2. Identified rat microsomal polypeptides which comigrate exactly with hamster polypeptides are NADPH–cytochrome P-450 reductase (streak B), actin (spot L), cytochrome P-450d (streak I), and cytochrome P-450 PB-1 (spot

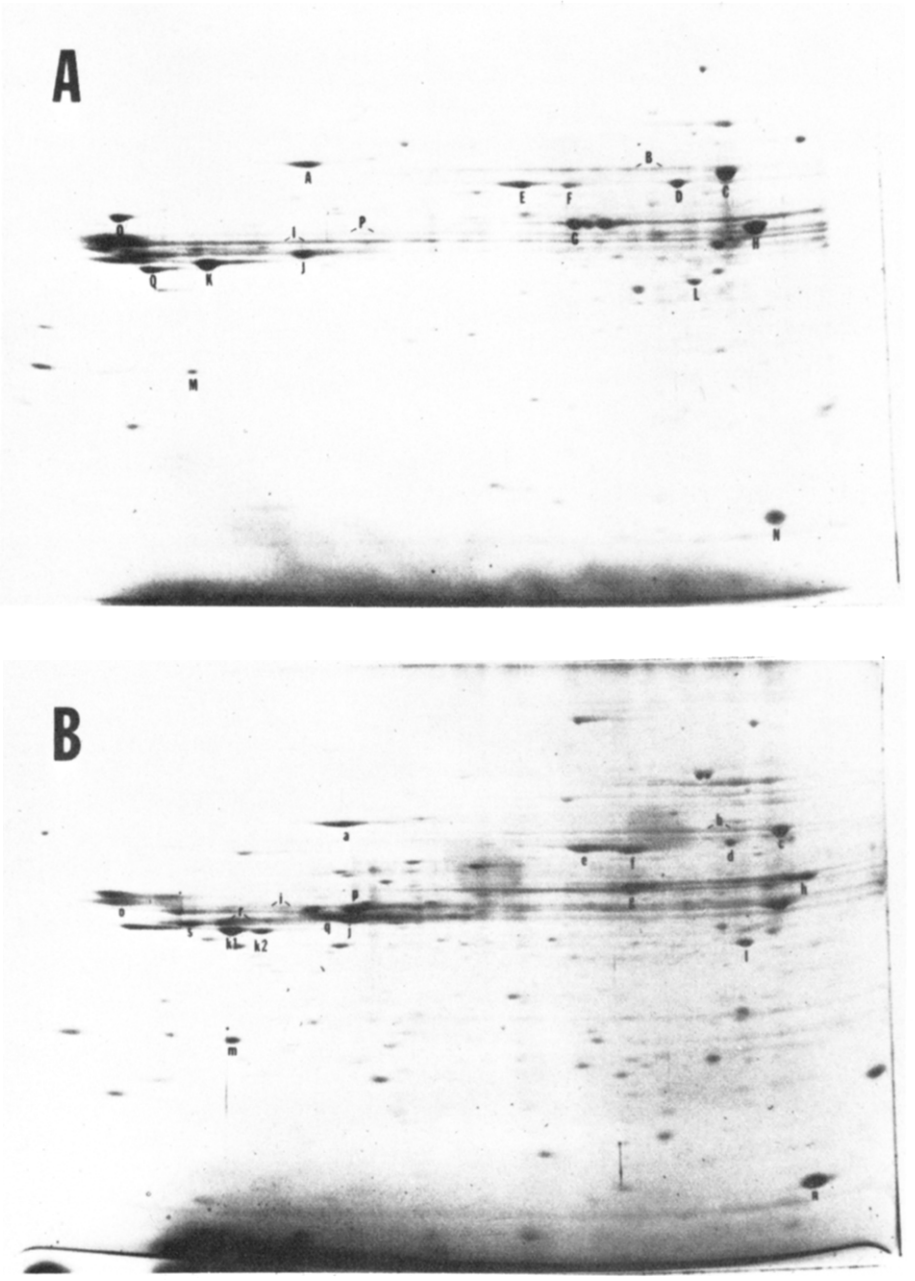

FIGURE 1: Two-dimensional electrophoretograms for hepatic microsomes from β-naphthoflavone-treated rats and hamsters. (A) Microsomes from β-napthoflavone-treated rats; (B) microsomes from β-naphthoflavone-treated hamsters. Isoelectric focusing was from right (acidic end) to left (basic end) and second-dimensional $NaDodSO_4$/polyacrylamide gel electrophoresis was from top to bottom. The designations for some major polypeptides in the rat (upper case) and the hamster (lower case) are described in Table II. Other experimental details are found under Materials and Methods.

J). Other identified rat polypeptides whose homologues in hamster microsomes appear as closely migrating electrophoretic variants include proalbumin (spot E), albumin (spot F), epoxide hydrolase (spot K), NADH–cytochrome b_5 reductase (spot M), and cytochrome b_5 (spot N). In general, the overall polypeptide patterns are quite similar for rat and hamster microsomes. However, some major polypeptides in either rats or hamsters may represent species-specific proteins since an electrophoretic homologue is not apparently present in microsomes from the other species (e.g., a homologue for rat polypeptide O does not appear to exist in hamster; see Figure 1).

A useful criterion for identifying hepatic microsomal polypeptides in IF/$NaDodSO_4$ gels as cytochromes P-450 or epoxide hydrolase is their induction as a result of various xenobiotic treatments (Vlasuk & Walz, 1982, 1980; Vlasuk et al., 1982a). Virtually all inducible polypeptides for both rats and hamsters are resolved in a limited, "variable" region of IF/SDS electrophoretograms for hepatic microsomes (see the boxed area in Figure 2). The only obvious exceptions to this finding are polypeptides A and a in rat and hamster, respectively, which increase after treating animals with 3-methylcholanthrene or β-naphthoflavone.[2] However, polypeptide A is a rat serum protein precursor.[3] Therefore, it is likely that all major, inducible forms of cytochromes P-450 and epoxide hydrolase in hamster microsomes are confined to this variable region of IF/$NaDodSO_4$ gels. For rat hepatic microsomes it has already been shown that all known cyto-

[2] The known induction of rat-hepatic NaDPH–cytochrome P-450 reductase by several of the agents tested is not very obvious in IF/$NaDodSO_4$ electrophoretograms since its polypeptide appears as a streak when resolved from microsomes (see polypeptide B in Figure 1A).

[3] J. Ghrayeb and F. G. Walz, Jr., unpublished experiments.

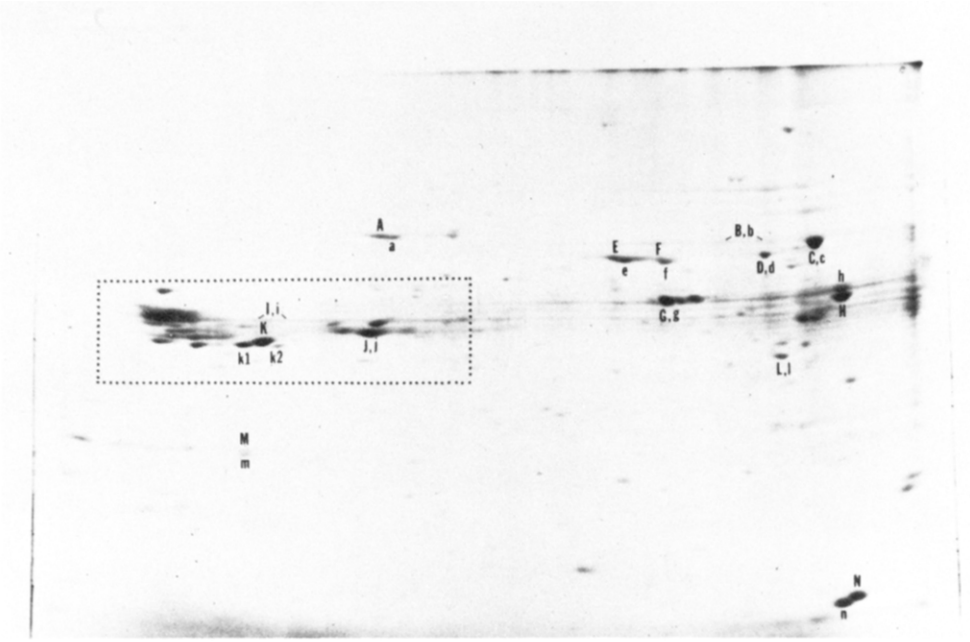

FIGURE 2: Two-dimensional coelectrophoretogram for hepatic microsomes from β-naphthoflavone-treated rats and hamsters. The microsomal samples used and the attitude of the electrophoretogram are the same as those in Figure 1. Designations are listed in Table II. The identifications of closely migrating rat and hamster polypeptides were accomplished by reference to the coordinates of adjacent polypeptides in Figure 1. Upper and lower case designations separated by a comma refer to rat and hamster polypeptides, respectively, which comigrate identically. In other cases, the positions of these designations reflect their relative migration. The boxed area is the "variable" region in which all known hepatic microsomal cytochromes P-450 and epoxide hydrolase are resolved (see text for details). Other experimental details are found under Materials and Methods.

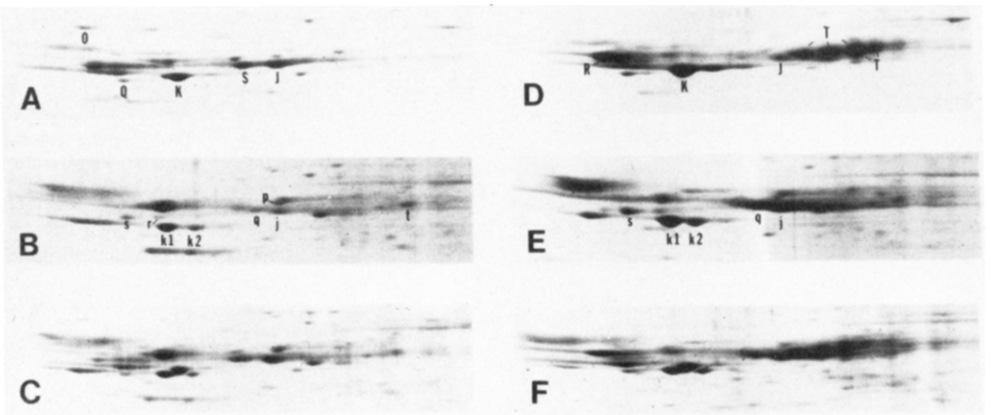

FIGURE 3: Portions of two-dimensional electrophoretograms for hepatic microsomes from untreated and phenobarbital-treated rats and hamsters. (A) Microsomes from untreated rats; (B) microsomes from untreated hamsters; (C) coelectrophoretogram for microsomes from untreated rats and hamsters; (D) microsomes from phenobarbital-treated rats; (E) microsomes from phenobarbital-treated hamsters; (F) coelectrophoretogram for microsomes from phenobarbital-treated rats and hamsters. The areas of the gels presented and their attitudes are the same as those shown for the boxed region in Figure 2. Designations are listed in Table II. Other conditions are found under Materials and Methods.

chromes P-450 tested and epoxide hydrolase are represented in this locale (Vlasuk & Walz, 1982, 1980; Vlasuk et al., 1982b). Considering these observations, only the variable regions of two-dimensional electrophoretograms for hepatic microsomes from control and xenobiotic-treated rats and hamsters are compared in Figures 3–5. To simplify consideration of these complex polypeptide patterns, attention is only given to hamster polypeptides which markedly change levels as a result of xenobiotic treatment and to rat polypeptides which were previously identified as cytochromes P-450 or epoxide hydrolase.

Appropriate portions of IF/NaDodSO$_4$ gels for analyzing hepatic microsomal polypeptides from control and phenobarbital-treated rats and hamsters are shown in Figure 3. Previous studies of rats (Vlasuk et al., 1982a) indicated that phenobarbital induced epoxide hydrolase, cytochrome P-450 PCN (Vlasuk et al., 1982b), cytochrome P-450 PB-1,[4] and members of a closely related multigene family of cytochromes P-450[5] (these are referred to as polypeptides K, R, J, and T, respectively, in Figure 3D). With the exceptions of epoxide hydrolase (spot K) and cytochrome P-450 PB-1 (spot J), all of these polypeptides were absent or present at barely detectable levels in microsomes from untreated rats (Figure 3A). In addition, the level of a putative male-specific cytochrome

[4] Hepatic cytochrome P-450 PB-1 was isolated from phenobarbital-treated rats (Waxman et al., 1982), and its ^{125}I-labeled tryptic peptide map exhibited complete homology with that for rat liver microsomal polypeptide PB2 (Vlasuk & Walz, 1980; Vlasuk et al., 1982a) which had been tentatively identified as an uncharacterized form of the enzyme (F. G. Walz, Jr., A. Rampersaud, and D. J. Waxman, unpublished experiments).

[5] In the colony of rats used these include cytochrome P-450b$_H$ (Ryan et al., 1982b), cytochrome P-450e (Ryan et al., 1982a), and their presumed allozymic variants (Walz et al., 1982).

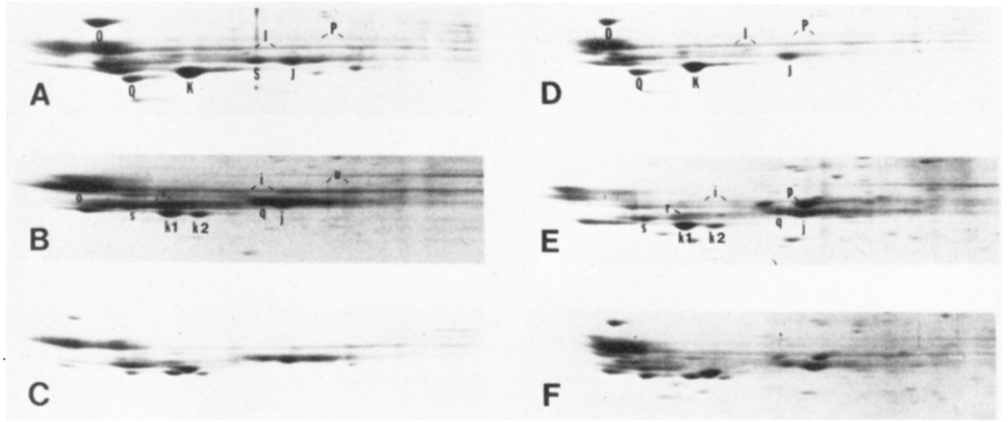

FIGURE 4: Portions of two-dimensional electrophoretograms for hepatic microsomes from 3-methylcholanthrene-treated and β-naphthoflavone-treated rats and hamsters. (A) Microsomes from 3-methylcholanthrene-treated rats; (B) microsomes from 3-methylcholanthrene-treated hamsters; (C) coelectrophoretogram of microsomes from 3-methylcholanthrene-treated rats and hamsters; (D) microsomes from β-naphthoflavone-treated rats; (E) microsomes from β-naphthoflavone-treated hamsters; (F) coeelctrophoretogram for microsomes from β-naphthoflavone-treated rats and hamsters. The areas of the gels presented and their attitudes are the same as those shown for the boxed region in Figure 2. Designations are listed in Table II. Other conditions are found under Materials and Methods.

P-450 [polypeptide M2 (Vlasuk & Walz, 1982)] was decreased as a result of phenobarbital treatment (compare polypeptide S in Figure 3A,D). In contrast with this complex induction program in rats, the effect of phenobarbital treatment of hamsters was only to increase the levels of polypeptides that were obviously present in control microsomes, and these include polypeptides j, k_1, k_2, q, and s (compare Figure 3B,E). When microsomes from corresponding rats and hamsters were coelectrophoresed, it was found that rat polypeptide J exactly comigrated with hamster polypeptide j but that phenobarbital-induced rat polypeptides T and R had no apparent homologues in hamster microsomes.

IF/NaDodSO$_4$ gel electrophoretic analysis of hepatic microsomes from 3-methylcholanthrene-treated rats was previously reported (Vlasuk & Walz, 1980, 1982) and indicated that a putative cytochrome P-450 (polypeptide MC1) and cytochromes P-450a, P-450c, and P450d were significantly induced by this xenobiotic (see rat polypeptides O, Q, P, and I, respectively, in Figure 4A). In addition, cytochrome P-450 PB-1 and polypeptide M2 were somewhat decreased after 3-methylcholanthrene treatment (Vlasuk & Walz, 1982; compare polypeptides S and J, respectively, in Figures 3A and 4A). Polypeptides MC1 (spot O) and cytochrome P-450a (spot Q) were found in microsomes from untreated rats (Figure 3A), whereas cytochrome P-450c and P-450d were not present at significant levels in control microsomes (Thomas et al., 1981; Vlasuk & Walz, 1980; Vlasuk et al., 1982b). The present results indicate that β-naphthoflavone treatment of rats gives essentially the same results as those for 3-methylcholanthrene (compare Figure 4A,D) and that neither of these agents induces polypeptides in common with phenobarbital. Six hepatic microsomal polypeptides are significantly induced by 3-methylcholanthrene treatment of hamsters, and these include polypeptides i, j, o, q, r, and s (Figure 4B), whereas the induction of streaked polypeptide u is less certain. Previous results using one-dimensional NaDodSO$_4$/polyacrylamide gel electrophoresis to analyze hepatic microsomal polypeptides from 3-methylcholanthrene-treated hamsters showed increases in polypeptide bands having apparent molecular weight values of 54000 and 49000 (Thorgeirsson et al., 1979) which probably reflect increases in polypeptide i and polypeptides r plus s, respectively (see Figure 4B). Hamster polypeptides o, i, and u were not obviously present in microsomes from untreated animals (Figure 3B), whereas polypeptide p which is present in control microsomes was not detectable after 3-methylcholanthrene treatment. Unlike rats, the pattern of microsomal polypeptides in hamsters after treatment with β-naphthoflavone is considerably different from that for 3-methylcholanthrene (compare Figure 4B,E). Both of these xenobiotics induced polypeptides i, j, q, r, and s, but polypeptides i, r, and s were increased to a lesser extent after β-naphthoflavone treatment. In addition, the level of polypeptide o which is dramatically increased by 3-methylcholanthrene does not appear to be induced by β-naphthoflavone. However, β-naphthoflavone had the unique effect of increasing the level of polypeptide p which appeared to be repressed by 3-methylcholanthrene treatment. Coelectrophoresis of rat and hamster microsomes from animals treated with either 3-methylcholanthrene (Figure 4C) or β-naphthoflavone (Figure 4F) indicated that hamster polypeptide i exactly comigrated as a streak with rat polypeptide I (cytochrome P-450d) and that hamster polypeptide u exactly comigrated with rat polypeptide P (cytochrome P-450c).

The pattern of hepatic microsomal polypeptides after *trans*-stilbene oxide treatment of rats has already been shown to be qualitatively the same as that for phenobarbital (Vlasuk et al., 1982b) (compare Figures 3D and 5A).[6] The most noticeable effect following *trans*-stilbene oxide treatment of hamsters was the increased levels of polypeptides k_1 and k_2, whereas barely significant increases were observed for polypeptides j, q, and t (compare Figures 3B and 5B).

The major hepatic microsomal polypeptide induced in rats by pregnenolone-16α-carbonitrile is polypeptide R which has been identified (Vlasuk et al., 1982b) as cytochromes P-450 PCN (Elshourbagy & Guzelian, 1980) (compare Figures 3A and 5D). In contrast, treatment of hamsters with this agent had no effect on the pattern of microsomal polypeptides (compare Figures 5E and 3B).

Considering that electrophoretically related polypeptides in hepatic microsomes from hamsters and rats might have similar primary structures, it was hoped that ^{125}I-labeled tryptic peptide mapping of selected rat and hamster polypeptides resolved in IF/NaDodSO$_4$ gels could further substantiate the tentative identification of unknown hamster cytochromes P-450 and epoxide hydrolases by revealing structural homologies with identified rat forms of these enzymes. Hamster polypeptides

[6] The different arrays of polypeptides T in Figures 3D and 5A are due to allozymic variation in the pooled rat samples used in this study (Walz et al., 1982).

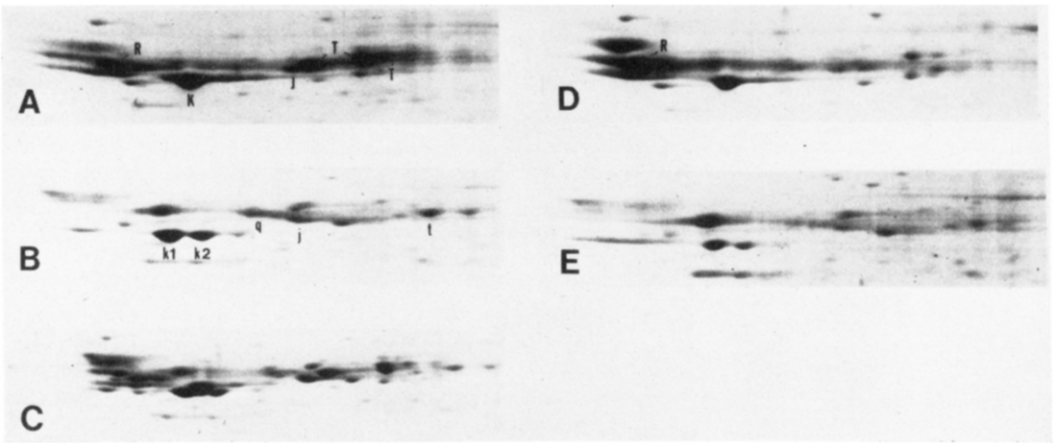

FIGURE 5: Portions of two-dimensional electrophoretograms for hepatic microsomes from *trans*-stilbene oxide treated and pregnenolone-16α-carbonitrile-treated rats and hamsters. (A) Microsomes from *trans*-stilbene oxide treated rats; (B) microsomes from *trans*-stilbene oxide treated hamsters; (C) coelectrophoretogram for microsomes from *trans*-stilbene oxide treated rats and hamsters; (D) microsomes from pregnenolone-16α-carbonitrile-treated rats; (E) microsomes from pregnenolone-16α-carbonitrile-treated hamsters. The areas of the gels presented and their attitudes are the same as those shown for the boxed region in Figure 2. Designations are listed in Table II. Other conditions are found under Materials and Methods.

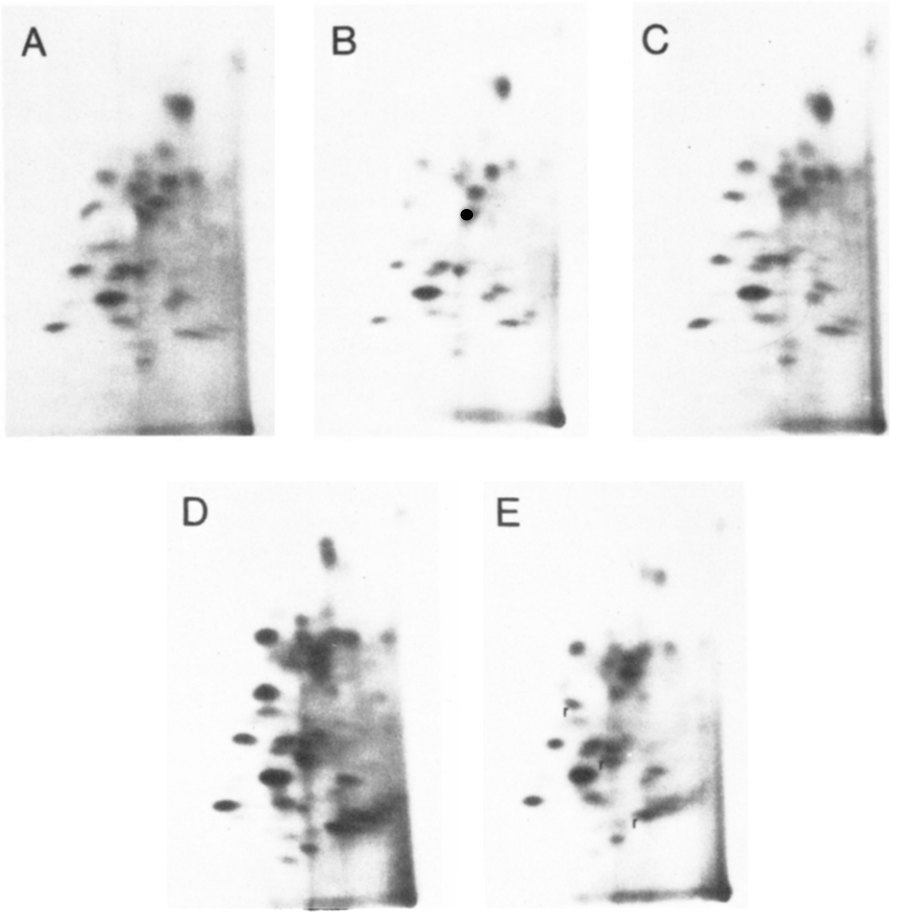

FIGURE 6: Radioiodinated tryptic peptide maps of hepatic microsomal polypeptides k_1 and k_2 from hamsters and rat hepatic microsomal epoxide hydrolase. (A) Hamster polypeptide k_1; (B) hamster polypeptide k_2; (C) mixture of hamster polypeptides k_1 and k_2; (D) rat-hepatic microsomal epoxide hydrolase (polypeptide K); (E) mixture of rat epoxide hydrolase and hamster polypeptide k_1. Polypeptides were excised from Coomassie blue stained IF/NaDodSO$_4$ gels for rat and hamster microsomes. Origins of the maps are in the lower right corners. Electrophoresis was from the right (anode) to the left (cathode), and chromatography was from bottom to top. The designation r refers to peptides characterizing rat epoxide hydrolase which are not found for hamster polypeptides k_1 and k_2. Other conditions were as described under Materials and Methods.

k_1 and k_2 closely migrated with rat polypeptide K which was previously identified as epoxide hydrolase (Vlasuk et al., 1982a,b). Even though polypeptides k_1 and k_2 migrated to slightly different coordinates in both dimensions of these gels, their peptide maps were identical (Figure 6A–C). These, in turn, were extremely homologous with those for rat-microsomal polypeptide K (epoxide hydrolase) (Figure 6D). In fact, the only species-related differences in these peptide maps were three additional peptides for rat polypeptide K (Figure 6E). Rat microsomal polypeptide J was recently identified as cytochrome P-450 PB-1 (Waxman et al., 1982).[4] This polypeptide exactly comigrated in IF/NaDodSO$_4$ gels with hamster polypeptide j (see above). However, in this case the peptide map for hamster polypeptide j evidenced ~45%

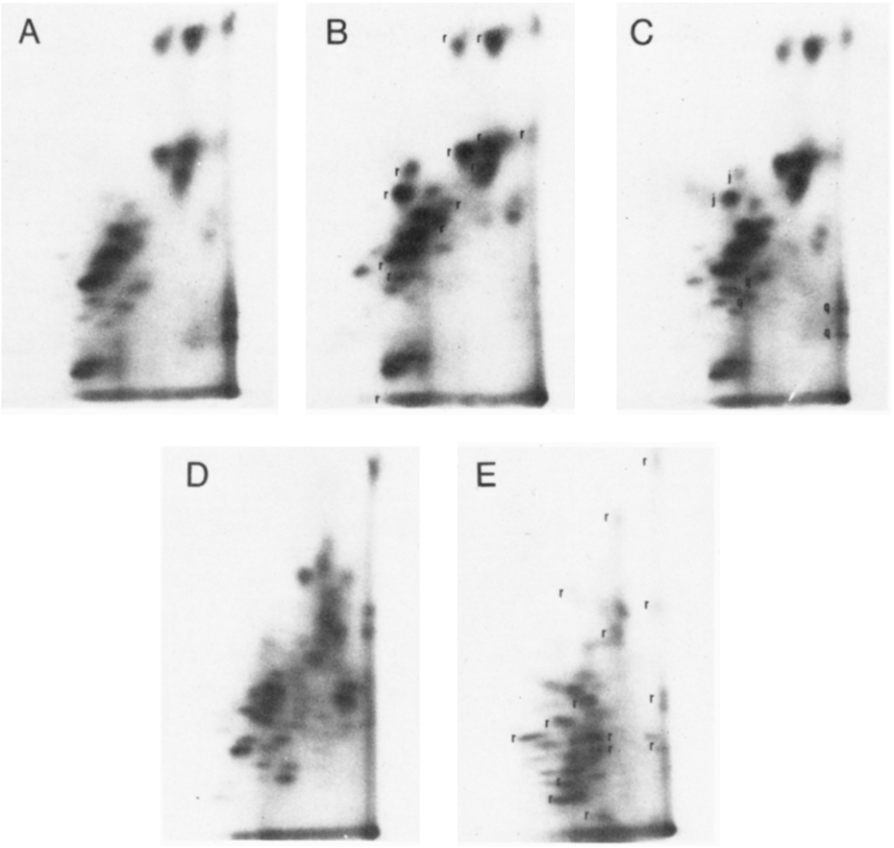

FIGURE 7: Radioiodinated tryptic peptide maps for putative hamster liver cytochromes P-450 resolved in IF/NaDodSO$_4$ gels of hepatic microsomes. (A) Hamster polypeptide q; (B) hamster polypeptide j; (C) mixture of hamster polypeptides j and q; (D) hamster polypeptide p; (E) hamster polypeptide i. The peptide designations r for hamster polypeptide j refer to peptides in common with rat polypeptide J. The peptide designations j and q in the peptide map for a mixture of polypeptides j and q are unique to their respective polypeptides. The peptide designations r for hamster polypeptide i refer to peptides in common with rat polypeptide I. The attitudes of these maps are the same as those in Figure 6, and other details are found under Materials and Methods.

homology with rat polypeptide J (Figure 7B). Rat microsomal polypeptide I [cytochrome P-450d (Ryan et al., 1980)] co-migrated exactly with hamster polypeptide i after two-dimensional gel electrophoresis (see above). In this case, the peptide map homology between rat and hamster forms was ~40% (Figure 7E). Initial attempts to establish other fingerprint homologies involved hamster polypeptides q (Figure 7A) and p (Figure 7D). A close relationship of polypeptides q and j is revealed in Figure 7C where only six peptide differences (i.e., out of ~27 peptide spots) were detected. On the other hand, very few homologous peptides were observed in the peptide map for hamster polypeptide p when compared with other hamster polypeptides or rat polypeptides T (Vlasuk et al., 1982a).

Discussion

The *relative* levels of cytochrome P-450 in hepatic microsomes from control, 3-methylcholanthrene-treated, and β-naphthoflavone-treated rats and hamsters shown in Table I agree with those reported previously (Gingell et al., 1981). Significantly greater specific contents of cytochrome P-450 characterized microsomes from untreated hamsters vis-à-vis those from untreated rats. Other differences between these species were observed after treatments with *trans*-stilbene oxide or pregnenolone-16α-carbonitrile which were reasonably good inducers of hepatic microsomal cytochromes P-450 in rats but had marginal or no effect, respectively, on the level of these hemoproteins in hamster liver.

A major purpose of the present study was to identify some hamster cytochromes P-450 and epoxide hydrolases which are resolved as polypeptides in IF/NaDodSO$_4$ gels of hepatic microsomes. Such identifications are necessarily tentative since purified forms of cytochromes P-450 and epoxide hydrolase from hamsters are not yet available for definitive comparisons. The minimal evidence previously used to tentatively identify rat liver forms of these enzymes in IF/NaDodSO$_4$ gels included (1) polypeptide size (apparent molecular weight values from 45 000 to 60 000), (2) polypeptide charge (apparent p*I* values ≥7), and (3) polypeptide inducibility by xenobiotics (Vlasuk & Walz, 1982, 1980; Vlasuk et al., 1982a). Tentative identifications of rat liver microsomal polypeptides based on this evidence were subsequently verified for epoxide hydrolase and eight distinct forms of cytochrome P-450 (Vlasuk, et al., 1982a,b).[4] Therefore, it seemed appropriate to apply the same criteria for categorizing hepatic microsomal polypeptides from hamsters, understanding that noninducible forms of cytochrome P-450 (and epoxide hydrolase) would be excluded from this preliminary survey. A list of hamster polypeptides that might represent cytochromes P-450 or epoxide hydrolase and their relative levels in microsomes after pretreatment with different xenobiotics are presented in Table III.

The identification of hamster polypeptides k_1 and k_2 as two forms of hepatic microsomal epoxide hydrolase is strongly supported by their extensive peptide map homology with the corresponding rat enzyme (Figure 6). The structural similarity of epoxide hydrolase from hamster and rat hepatic microsomes was previously indicated by cross-reaction of antibody to the rat enzyme with hepatic microsomes from hamsters (Levin et al., 1978). Furthermore, rat epoxide hydrolase is markedly induced by phenobarbital and *trans*-stilbene oxide (Thomas

Table III: Relative Levels of Inducible Polypeptides in Hamster Liver Microsomes Which Are Tentatively Identified as Cytochromes P-450 or Epoxide Hydrolase

polypeptide	relative levels[a] control[b]	PB	MC	BNF	TSO
i	0	0	2	1	0
j	1	3	2	2	2
k_1	1	2	1	1	3
k_2	1	2	1	1	3
o	1	2	3	2	1
p	1	1	0	2	1
q	1	3	2	2	2
r	1	2	3	2	1
s	1	2	3	2	1
t	1	1	1	1	1
u	0	0	t	0	0

[a] Relative levels were determined from Coomassie blue stained IF/NaDodSO$_4$ gels (Figures 1–5) and were judged independently for each polypeptide; numbers only distinguish "greater than" values, t indicates trace amounts, and 0 indicates none detectable. [b] Control, PB, MC, BNF, and TSO refer to microsomes from untreated hamsters and those treated with phenobarbital, 3-methylcholanthrene, β-naphthoflavone, and *trans*-stilbene oxide, respectively. The results for pregnenolone-16α-carbonitrile-treated hamsters were the same as those for control animals.

et al., 1981) which also appears to be true for hamster polypeptides k_1 and k_2. Whether hamster polypeptides k_1 and k_2 are the products of one or two genes is currently unknown. However, two forms of hamster microsomal epoxide hydrolase were previously indicated by Ouchterlony analysis using anti-rat epoxide hydrolase which yielded two immunoprecipitin bands for hamster microsomes and one band for rat microsomes (Levin et al., 1978). A previous comparison of rat and human hepatic microsomal epoxide hydrolases revealed considerable homology in their NH_2-terminal sequences as well as their peptide maps (Du Bois et al., 1982). Therefore, it appears that the primary structure of this enzyme is highly conserved among these mammalian species.

The circumstantial case that polypeptide j represents a cytochrome P-450 is compelling since it was the major induced polypeptide in hepatic microsomes from phenobarbital-induced hamsters which were also characterized by an increased cytochrome P-450 content of 1.7 nmol/mg of protein. Furthermore, the structure of this polypeptide is closely related to that for hamster polypeptide q as evidenced by peptide mapping (Figure 7A–C), and the combined levels of polypeptides j and q in microsomes from phenobarbital-treated hamsters quantitatively represent virtually all of the increased polypeptide content of these microsomes, exclusive of epoxide hydrolase (polypeptides k_1 and k_2). This correlation is pertinent since the *increased* content of cytochrome P-450 resulting from phenobarbital induction should represent at least 8.5% of the total microsomal protein.[7] It is interesting that hamster polypeptide j exactly comigrates in IF/NaDodSO$_4$ gels with cytochrome P-450 PB-1 (rat polypeptide J). A structural relationship between these rat and hamster polypeptides was evidenced by a ~45% homology in their ^{125}I-labeled tryptic peptide maps (Figure 7B). Considering these results it is tentatively concluded that hamster polypeptides j and q are related forms of cytochrome P-450. It is noteworthy that these polypeptides are coinduced by all of the xenobiotics tested which induce cytochromes P-450 (Table III).

[7] Calculated for an increase of 1.7 nmol of cytochrome P-450/mg of protein assuming a molecular weight value of 50 000.

Members of a closely related multigene family of phenobarbital-inducible cytochromes P-450 in rat liver (Vlasuk et al., 1982a; Walz et al., 1982; see polypeptides T in Figures 3D and 5A) do not appear to have homologues in the hamster. This seems to be true since phenobarbital or any of the other xenobiotics tested did not induce hamster polypeptides that migrate to the discrete region of IF/NaDodSO$_4$ gels where rat polypeptides T are resolved (Figures 3–5). Furthermore, those major hamster polypeptides adjacent to this region (i.e., polypeptides j, q, and p) had peptide maps (Figure 7A–C) which were completely different from the common pattern found for polypeptides T (Vlasuk et al., 1982a). Nevertheless, the possibility that some minor, noninducible hamster polypeptides are homologous with this closely related multigene family in rats cannot be excluded at this time.

Hepatic cytochrome P-450 PCN (polypeptide R) is also induced by phenobarbital in rats (Heuman et al., 1982; Vlasuk et al., 1982b). This hemoprotein was first isolated from animals treated with pregnenolone-16α-carbonitrile and represents the major induced polypeptide in hepatic microsomes from rats treated with this agent (Elshourbagy & Guzelian, 1980; Vlasuk et al., 1982b; see Fig. 5D). It appears that an inducible hamster homologue of this enzyme does not exist since pregenolone-16α-carbonitrile treatment had no effect on either the cytochrome P-450 content or polypeptide composition of hamster liver microsomes. Furthermore, no induced hamster polypeptide in hepatic microsomes from any of the xenobiotic-treated animals appeared to be electrophoretically related to cytochrome P-450 PCN in IF/NaDodSO$_4$ gels. However, the existence of a noninducible hamster homologue to rat cytochrome P-450 PCN or an inducible homologue that migrates very differently in IF/NaDodSO$_4$ gels (e.g., polypeptide o) cannot be ruled out at this time.

Hamster polypeptide i shares the following characteristics with rat polypeptide I [cytochrome P-450d (Ryan et al., 1980)]: (1) both polypeptides exactly comigrate as streaks in IF/NaDodSO$_4$ gels, (2) both polypeptides are not detectable in IF/NaDodSO$_4$ gels for microsomes from untreated animals, and (3) both polypeptides are only induced by 3-methylcholanthrene and β-naphthoflavone among the agents tested. In addition, the peptide maps for hamster polypeptide i and rat polypeptide I have ~40% homology. These results strongly suggest that hamster polypeptide i is a homologue of rat liver cytochrome P-450d. On the other hand, the existence of a hamster homologue for rat liver cytochrome P-450c (polypeptide P) is less certain since hamster polypeptide u which exactly comigrates with rat polypeptide P is only barely detectable in IF/NaDodSO$_4$ gels for microsomes from 3-methylcholanthrene-treated animals and could not be submitted to peptide mapping.

The homologies in peptide maps comparing putative hamster cytochromes P-450 and their electrophoretically identical rat homologues (Figure 7B,E) appear to be sufficient to establish structural relatedness but were lower than expected. It is possible that this resulted from the presence of different, unresolved hamster polypeptides in the samples used for fingerprinting a given Coomassie blue stained locale of the IF/NaDodSO$_4$ gel. However, this does not appear to be the case since the same peptide map was observed for polypeptide j regardless of its relative amount in IF/NaDodSO$_4$ electrophoretograms for microsomes from control and phenobarbital-treated hamsters (data not shown). In addition, the Coomassie blue stained area which was sampled for peptide mapping of polypeptide i was devoid of staining material in IF/NaDodSO$_4$ gels for microsomes from untreated hamsters.

Furthermore, all purified forms of rat liver cytochrome P-450 which have been tested to date gave the same fingerprint as their corresponding polypeptides which were resolved directly from microsomes in IF/NaDodSO$_4$ gels.[8] Therefore, in contrast to epoxide hydrolase, it seems that related cytochromes P-450 in rats and hamsters may have considerably different primary structures even though they are electrophoretically identical in two-dimensional IF/NaDodSO$_4$ gels. If these apparently related cytochromes P-450 are indeed as structually diverse as suggested by their peptide fingerprints, this could reflect an unusual genetic adaptability and/or ecological pressure for their genes over the course of evolution.

The results discussed above suggest that hamsters and rats have overlapping but not identical ensembles of cytochrome P-450 structural genes. The absence of comparable genes in the hamster for rat liver cytochrome P-450 PCN and the multigene family of cytochromes P-450b and P-450e could explain some of the major differences between these species regarding their induced microsomal polypeptide patterns after phenobarbital or pregnenolone-16α-carbonitrile treatments. However, it is possible that homologous genes for these inducible hemoproteins exist in the hamster but are not reponsive to the xenobiotic agents tested in this study. Viewed from a different perspective, homologous forms of several putative cytochromes P-450 for the hamster (i.e., polypeptides p, r, s, and t) either may be absent in the rat or are not inducible by the agents tested to date. In any event, for both rats and hamsters approximately the same number of inducible hepatic microsomal polypeptides can be tentatively identified as cytochromes P-450 on the basis of circumstantial criteria. Additional fingerprint comparisons of hepatic microsomal polypeptides from rats and hamsters should distinguish some of these possibilities and provide an improved basis for judging the structural relatedness of liver cytochromes P-450 in these species.

As a generality, it appears that the regulation of hepatic microsomal polypeptides in the hamster is less strict than that for the rat. In fact, polypeptide i (and possibly polypeptide u) is the only inducible polypeptide which was not obviously present in IF/NaDodSO$_4$ gels for hepatic microsomes from untreated hamsters, whereas almost *all* of the inducible hepatic microsomal polypeptides including cytochromes P-450c, P-450d, P-450 PCN, and the multigene family of cytochromes P-450b and P-450e are not present at significant levels in IF/NaDodSO$_4$ gels for microsomes from untreated rats. On the other hand, similar induction specificities were observed for rat liver cytochrome P-450d (i.e., by 3-methylcholanthrene and β-naphthoflavone) and epoxide hydrolase (i.e., by phenobarbital and *trans*-stilbene oxide) vis-ă-vis their hamster liver homologues.

The preliminary conclusions of this initial survey of hamster liver epoxide hydrolase and cytochromes P-450 may be summarized as follows: (1) two forms of hamster liver microsomal epoxide hydrolase exist whose two-dimensional ^{125}I-labeled peptide maps are identical with each other and ~90% homologous with that for the corresponding, electrophoretically distinct rat liver enzyme; (2) two putative hamster liver cytochromes P-450 (polypeptides j and p) appear to have markedly different primary structures compared to their corresponding rat forms (i.e., only 40–45% peptide map homology) even though they exactly comigrate with them in two-dimensional IF/NaDodSO$_4$ gels; (3) two putative hamster liver cytochromes P-450 (polypeptides j and q) are closely related to each other having peptide maps which are ~80% homologous; (4) homologues of major, inducible rat-hepatic cytochromes P-450 [i.e., cytochrome P-450 PCN (Elshourbagy & Guzelian, 1980) and the multigene family of cytochromes P-450b (Ryan et al., 1979, 1982b) and P-450e (Ryan et al., 1982a)] do not appear to exist in hamsters; (5) in general, the hepatic microsomal levels of inducible cytochromes P-450 in the hamster are much less dependent on xenobiotic treatment than those in the rat; (6) the induction program for hepatic microsomal polypeptides in rats is the same for either 3-methylcholanthrene or β-naphthoflavone, whereas in hamsters the patterns of induced polypeptides are clearly distinct after treatment with these xenobiotics; (7) effective xenobiotic inducers for either cytochrome P-450d or epoxide hydrolase in rat liver are the same as those for their corresponding homologues in hamster liver, whereas the induction specificities for homologous forms of a putative, hepatic cytochrome P-450 (i.e., polypeptides Q and q for rats and hamsters, respectively) are clearly different. At this stage of the investigation, it seems that the ensemble of hepatic cytochrome P-450 structural genes and their regulation have undergone a considerable diversification between rats and hamsters. This is sharply contrasted with the structural and regulatory conservation observed for hepatic microsomal epoxide hydrolase in these two species.

[8] These include cytochromes P-450a, P-450b$_{LE}$, P-450c (Ryan et al., 1979), P-450b$_H$ (Ryan et al., 1982b), P-450d (Ryan et al., 1980), P-450e (Ryan et al., 1982a), and P-450 PB-1 (Waxman et al., 1982).

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Polymerization of the Tubulin–Colchicine Complex: Relation to Microtubule Assembly†

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ABSTRACT: The polymerization of purified tubulin–colchicine complex, which results in polymers different from microtubules under microtubule-promoting conditions, has been characterized. It proceeds as a nucleated condensation polymerization, requires Mg^{2+}, and is inhibited by small concentrations of Ca^{2+}. Polymerization requires GTP binding, but GDP is inhibitory. The GTPase activity proceeds, but it is unlinked to polymerization. The thermodynamic characteristics of the growth reaction, namely, the apparent changes of free energy, enthalpy, entropy, heat capacity, and preferential interaction with H^+ and Mg^{2+}, are very similar to those of microtubule assembly. It is proposed that the interactions responsible for the two types of polymerization are very similar and that the molecular mechanism of microtubule inhibition by colchicine may consist in a drug-induced distortion of the normal protomer bonding geometry.

Colchicine has been used for many years as an inhibitor of mitosis (Dustin, 1978). In 1968 Weisenberg et al. (1968) purified the protein tubulin, which is the major constituent of microtubules and the main cellular receptor of colchicine, and to which the alkaloid binds tightly (Wilson & Bryan, 1974). It has been well established that colchicine inhibits substoichiometrically mitosis (Taylor, 1963) and the assembly of microtubules in vitro (Olmsted & Borisy, 1973). Microtubule assembly in vitro conforms to a nucleated helical condensation polymerization mechanism (Gaskin et al., 1974; Lee & Timasheff, 1975, 1977), just like the assembly of filamentous actin (Oosawa & Asakura, 1975). Margolis & Wilson (1977) have shown in their study of steady-state microtubules assembled in vitro that addition of tubulin promoters occurs preferentially at one end of the polymer and release preferentially at the other. This process, which is accompanied by the hydrolysis of bound GTP at the growing end (Carlier & Pantaloni, 1981), results in a net flow of promoters through the constant size polymer. The flow through, or treadmilling, is poisoned by colchicine which acts at the ends (Margolis & Wilson, 1977, 1978; Margolis et al., 1980), leading to the proposal that the tubulin–colchicine complex binds tightly to the growing end of microtubules and inhibits kinetically the addition of further promoters (Margolis & Wilson, 1977). On the other hand, a study of the effects of the tubulin–colchicine complex on the non-steady-state seeded assembly of microtubules has led to the conclusion that tubulin bound to colchicine copolymerizes with unliganded tubulin, reducing the affinity of the polymer for new protomers (Sternlicht & Ringel, 1979).

In a kinetic study of the effects of the tubulin–colchicine complex on small amplitude growth of microtubules, Lambeir & Engelborghs (1980) have found that the binding of tubulin–colchicine to microtubules inhibits growth, is rapid and reversible, and proceeds with an estimated association constant of the same order of magnitude as that of unliganded tubulin. The particular mechanisms of the inhibition of assembly proposed in each of the above studies are likely the consequence of the different experimental conditions used and of the approaches applied to a complex problem and need not necessarily be contradictory.

It has been recognized that the precise mechanism by which tubulin–colchicine terminates growth is not known (Margolis & Wilson, 1981; Lambeir & Engelborghs, 1980). Which are then the basic molecular phenomena that result in the inhibition of polymerization? It is clear that, for inhibition of the assembly of the normal polymer, colchicine binding has to cause changes in the normal protein–protein interactions between protomers. Knowledge of the nature of intermolecular contacts formed and of the detailed geometries of the normal and drug-altered tubulin additions to a growing microtubule should most likely lead to an understanding of the basic mo-

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