Polypeptide Patterns of Hepatic Microsomes from Long-Evans Rats Treated with Different Xenobiotics†

George P. Vlasuk, Dene E. Ryan, Paul E. Thomas, Wayne Levin, and Frederick G. Walz, Jr.*

ABSTRACT: Two-dimensional gel electrophoresis was used to analyze hepatic microsomal polypeptides after treatment of immature, male Long-Evans rats with 3-methylcholanthrene, pregnenolone-16α-carbonitrile, isosafrole, SKF-525A, Aroclor-1254, γ-chlordane, or *trans*-stilbene oxide. Epoxide hydrolase and cytochromes P-450a, P-450b_{LE}, P-450c, P-450d, and P-450e were all identified as resolved polypeptides in these electrophoretograms. Idiosyncratic polypeptide patterns characterized the microsomal preparations following treatment of rats with each inducing agent. Immunochemically identical cytochromes P-450b_{LE} and P-450e were always present at the same relative levels even though their total amount varied 3-fold after induction by isosafrole, SKF-525A, Aroclor-1254, γ-chlordane, and *trans*-stilbene oxide. Cytochromes P-450c and P-450d were coinduced by 3-methylcholanthrene, isosafrole, and Aroclor-1254, but their relative amounts varied. Pregnenolone-16α-carbonitrile treatment resulted in an increase of a single major microsomal polypeptide which was also induced by phenobarbital, isosafrole, SKF-525A, Aroclor-1254, and *trans*-stilbene oxide. Only one polypeptide was identified as epoxide hydrolase in all of the microsomes analyzed. The results suggest that the levels of cytochromes P-450b_{LE} and P-450e may be subject to coordinate control, whereas the other cytochromes P-450 are independently regulated.

The oxidative metabolism of a given xenobiotic in mammalian liver is often catalyzed by the action of one or several hemoproteins, collectively designated cytochromes P-450, which are terminal enzymes of the microsomal mixed function monooxygenase system. The capacity of this system to recognize and metabolize a large number of chemically diverse substrates results from the existence of different cytochromes P-450, each of which exhibits a limited but overlapping substrate specificity (Lu & Levin, 1974). In addition, the total level of cytochromes P-450 in the hepatic endoplasmic reticulum can an be dramatically increased by treating animals with various xenobiotics which induce specific forms of these enzymes (Conney, 1967). At this time, at least seven distinct inducible hepatic cytochromes P-450 have been purified to homogeneity from rats treated with either phenobarbital (Guengerich, 1978; Ryan et al., 1979), 3-methylcholanthrene (Guengerich, 1978; Ryan et al., 1979), Aroclor-1254 (Ryan et al., 1979, 1982a,b), isosafrole (Ryan et al., 1980; Fisher et al., 1981), pregnenolone-16α-carbonitrile (Elshourbagy & Guzelian, 1980), or clofibrate (Gibson et al., 1982).

Recent efforts to characterize the population of induced cytochromes P-450 in rat liver have involved quantitative studies of several isozymes using immunological methods (Thomas et al., 1981; Heuman et al., 1982). The amounts of three immunochemically distinct forms of cytochrome P-450 in hepatic microsomes from control rats or rats treated with eight different inducing agents accounted for ~10% to ~90% of the total microsomal content, depending on the particular treatment (Thomas et al., 1981). It was also shown that different xenobiotics significantly induce the same forms of the enzyme [e.g., Aroclor-1254 treatment increased the levels of cytochromes P-450b and P-450c which are the major forms of the enzyme induced by phenobarbital and 3-methylcholanthrene, respectively (Thomas et al., 1981)]. A shortcoming of this approach is that cytochromes P-450b and P-450e could not be individually quantified since they appear to be immunochemically identical (Vlasuk et al., 1982; Ryan et al., 1982a). Furthermore, only those forms of cytochrome P-450 for which antibodies have been prepared can be assayed by this technique.

A complementary strategy for investigating cytochrome P-450 induction patterns is to analyze the polypeptide composition of liver microsomes by using two-dimensional IF/$NaDodSO_4$ gel electrophoresis[1] (Vlasuk & Walz, 1980). This method has already been employed to elucidate multiple forms of immunochemically identical phenobarbital-induced cytochromes P-450 in rat liver which vary with strain/colony of animal (Vlasuk et al., 1982) and to demonstrate that three of these forms [i.e., cytochromes P-450b_{LE} (Ryan et al., 1979), P-450b_H (Ryan et al., 1982a,b), and P-450e (Ryan et al., 1982a)] are genetically distinct products (Walz et al., 1982). Hepatic microsomes from phenobarbital-treated Blu-Long-Evans rats were characterized by cytochrome P-450b_{LE} and cytochrome P-450e which appeared in a ratio of at least 2:1, respectively (Vlasuk et al., 1982), and consititute ~57% of the total cytochrome P-450 content (Thomas et al., 1981).

The present study involved IF/$NaDodSO_4$ electrophoretic analysis of hepatic microsomes from Blu-Long-Evans rats after treatment with seven additional xenobiotics. The results indicate that the ratios of microsomal cytochromes P-450$_{LE}$ and P-450e are relatively constant (i.e., at least 2:1) regardless of whether they are induced by isosafrole, SKF-525A, Aroclor-1254, γ-chlordane or *trans*-stilbene oxide, even though their combined levels in microsomes varied approximately 3-fold (nanomoles per milligram of protein) after treatment with these agents (Thomas et al., 1981).[2] It was also found that

† From the Department of Chemistry, Kent State University, Kent, Ohio 44242 (G.P.V. and F.G.W.), and the Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche, Inc., Nutley, New Jersey 07110 (D.E.R., P.E.T., and W.L.). *Received June 4, 1982.* Supported in part by National Science Foundation Grant PCM-8107924.

[1] Abbreviations: IF/$NaDodSO_4$ gel electrophoresis, two-dimensional gel electrophoresis with isoelectric focusing in the first dimension and sodium dodecyl sulfate polyacrylamide gel electrophoresis in the second dimension; $NaDodSO_4$, sodium dodecyl sulfate; Blu-Long-Evans rats, Long-Evans rats from a colony at Blue Spruce Farms, Inc.; Cr-Holtzman rats, Holtzman rats from a colony at the Charles River Breeding Laboratories, Inc.; cytochrome P-450-PCN, a specific form of hepatic cytochrome P-450 isolated from pregnenolone-16α-carbonitrile-treated rats (Elshourbagy & Guzelian, 1980).

0006-2960/82/0421-6288$01.25/0

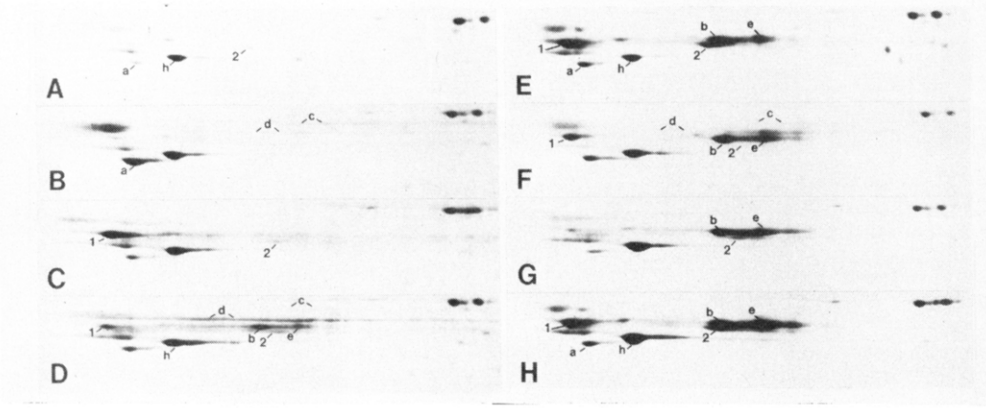

FIGURE 1: Portions of IF/NaDodSO$_4$ electrophoretograms for microsomes from sexually immature, male rats which were treated with different xenobiotics. Blu-Long-Evans rats were used. The gel portions shown represent a pH range in first-dimensional isoelectric focusing from ~8.0 to ~6.4, left to right, respectively, and a molecular weight region in second-dimensional NaDodSO$_4$/polyacrylamide gel electrophoresis from ~63 000 to 40 000, top to bottom, respectively. (A) Microsomes from *untreated* rats; (B) microsomes from *3-methylcholanthrene*-treated rats; (C) microsomes from *pregnenolone-16α-carbonitrile*-treated rats; (D) microsomes from *isosafrole*-treated rats; (E) microsomes from *SKF-525A*-treated rats; (F) microsomes from *Aroclor-1254*-treated rats; (G) microsomes from *γ-chlordane*-treated rats; (H) microsomes from *trans-stilbene oxide* treated rats. 100 μg of microsomal protein and Coomassie blue staining were used in all cases. Designations a, b, c, d, and e refer to cytochromes P-450a, P-450b$_{LE}$, P-450c, P-450d, and P-450e, respectively. Designations h, 1, and 2 refer to epoxide hydrolase, polypeptide PB1, and polypeptide PB2, respectively, which are induced by phenobarbital (Vlasuk et al., 1982). For clarity, these designations are not repeated in all cases. For reference, the molecular weight values deduced from NaDodSO$_4$/polyacrylamide gel electrophoresis gels for cytochromes P-450a, P-450b$_{LE}$, and P-450c are 48 000, 52 000, and 56 000, respectively (Ryan et al., 1979). The polypeptide pattern for microsomes from phenobarbital-treated rats was qualitatively similar to that shown for SKF-525A (cf. Vlasuk et al., 1982). Other details are found under Materials and Methods.

cytochromes P-450c and P-450d were coinduced by 3-methylcholanthrene, isosafrole, and Aroclor-1254, but their relative levels in microsomes depended on the xenobiotic used. Finally, the major microsomal polypeptide induced by pregnenolone-16α-carbonitrile (Elshourbagy & Guzelian, 1980) was also significantly induced by phenobarbital, isosafrole, SKF-525A, Aroclor, and *trans*-stilbene oxide.

Materials and Methods

Chemicals. 3-Methylcholanthrene was purchased from Sigma Chemical Co. *trans*-Stilbene oxide and γ-chlordane were obtained from Aldrich Chemical Co. and Velsicol Chemical Corp., respectively. Pregnenolone-16α-carbonitrile was a gift from G. D. Searle and Co., and Aroclor-1254 (lot no. KC-12-638) was a gift from Monsanto Co. SKF-525A [2-(dimethylamino)ethyl 2,2-diphenylvalerate hydrochloride] was obtained from Smith, Kline, and French Co., and isosafrole was from Eastman Kodak Co. Corn oil was from Matheson Coleman & Bell Co.

Animals and Their Treatment. Xenobiotic treatments of sexually immature (50–60 g) male, Blu-Long-Evans rats (Blue Spruce Farms, Altamont, NY) and the preparation of hepatic microsomes were previously described (Thomas et al., 1981). The total cytochrome P-450 contents of these microsomes and their levels of epoxide hydrolase and some specific forms of cytochromes P-450 have already been reported (Thomas et al., 1981).

Electrophoresis and Peptide Fingerprinting. Two-dimensional IF/NaDodSO$_4$ gel electrophoresis of microsomes (Vlasuk & Walz, 1980) and in situ ^{125}I-labeled tryptic peptide fingerprinting of resolved microsomal polypeptides (Vlasuk et al., 1982) have already been described.

Purified Enzymes. Cytochromes P-450a, P-450b, P-450c (Ryan et al., 1979), P-450d (Ryan et al., 1980), P-450e (Ryan et al., 1982a), and epoxide hydrolase (Lu et al., 1975) were prepared from male, Blu-Long-Evans rats according to published procedures.

Results and Discussion

Polypeptide patterns for hepatic microsomes from untreated Blu-Long-Evans rats and those treated with 3-methylcholanthrene, pregnenolone-16α-carbonitrile, isosafrole, SKF-525A, Aroclor-1254, γ-chlordane, or *trans*-stilbene oxide are shown in Figure 1. The portions of IF/NaDodSO$_4$ gels presented represent the areas in which epoxide hydrolase and all known cytochromes P-450 tested to date are resolved. This was demonstrated by coelectrophoresis of purified enzymes (i.e., epoxide hydrolase and cytochromes P-450a, P-450c, and P-450d) with microsomes (data not shown) and by in situ fingerprinting of the resolved microsomal polypeptides. Fingerprints for identifying epoxide hydrolase and cytochrome P-450a in IF/NaDodSO$_4$ gels are presented in Figure 2 and those for cytochromes P-450c and P-450d are shown in Figure 3. Similar criteria for identifying cytochromes P-450b$_{LE}$ and P-450e were reported previously (Vlasuk et al., 1982). It was always observed that epoxide hydrolase and cytochromes P-450a, P-450b$_{LE}$, and P-450e were resolved as spots after IF/NaDodSO$_4$ electrophoresis of microsomes, whereas cytochromes P-450c and P-450d did not focus in the first dimension and appeared as horizontal streaks. Nevertheless, these two latter proteins were resolved from other microsomal polypeptides through at least 90% of these streaks as judged by fingerprinting different segments (data not shown). Since purified cytochromes P-450c and P-450d also migrated as streaks in IF/NaDodSO$_4$ gels when run alone (data not shown), it appears that this tendency to disperse during isoelectric focusing is an intrinsic property of their polypeptide chains.

The existence of two forms of microsomal epoxide hydrolase in rat liver has been proposed, and it was suggested that they are characterized by the same apparent molecular weight in NaDodSO$_4$/polyacrylamide gel electrophoresis gels but have different charge properties (Guengerich et al., 1979). However, we have consistently observed a single polypeptide spot for this protein after IF/NaDodSO$_4$ electrophoresis of hepatic

[2] The level of cytochromes P-450b$_{LE}$ plus P-450e in hepatic microsomes from isosafrole-treated, immature, male Blu-Long-Evans rats is approximately 0.4 nmol/mg of microsomal protein (unpublished results).

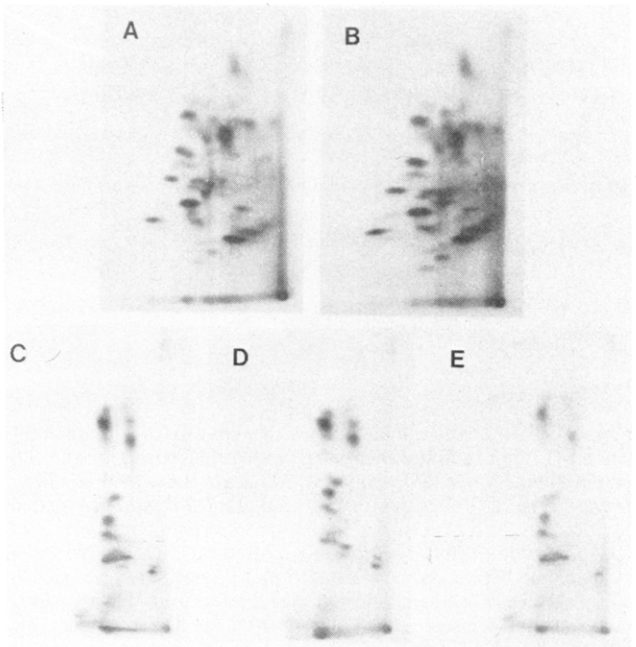

FIGURE 2: Radioiodinated peptide fingerprints of epoxide hydrolase and cytochrome P-450a from IF/NaDodSO$_4$ gels resolving Long-Evans rat liver microsomal polypeptides and purified forms of the enzymes. (A) Purified epoxide hydrolase; (b) polypeptide h (see Figure 1); (C) purified cytochrome P-450a; (D) polypeptide a (see Figure 1); (E) cofingerprint of polypeptide a and purified cytochrome P-450a. Polypeptides were excised from IF/NaDodSO$_4$ gels similar to those in Figure 1. 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. Approximately 5 × 10^5 cpm of ^{125}I-labeled peptides were applied in each case. In the cofingerprint, half of this amount of radioactivity was applied for each of the samples. Autoradiography was from 6 to 10 days at −70 °C by using Kodak SB-5 film.

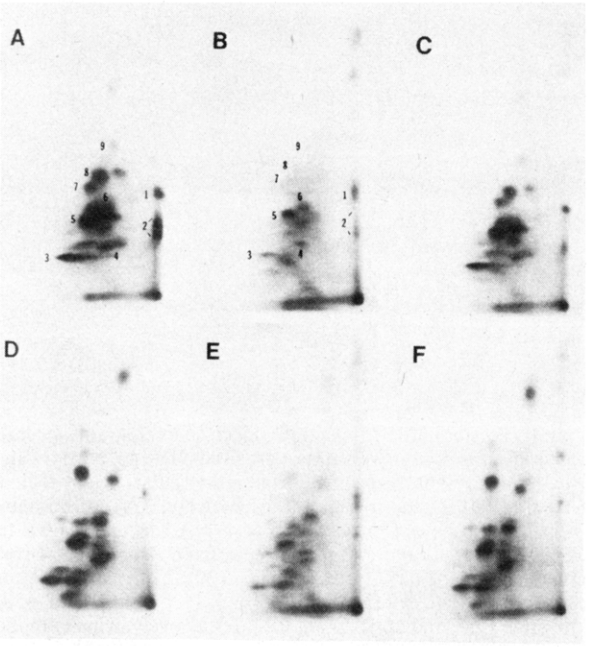

FIGURE 3: Radioiodinated peptide fingerprints of cytochromes P-450c and P-450d from IF/NaDodSO$_4$ gels resolving Long-Evans rat liver microsomal polypeptides and purified forms of the enzyme. (A) Purified cytochrome P-450c; (B) polypeptide c (see Figure 1); (C) cofingerprint of polypeptide c and purified cytochrome P-450c; (D) purified cytochrome P-450d; (E) polypeptide d (see Figure 1); (F) cofingerprint of polypeptide d and purified cytochrome P-450d. The portion of the polypeptide "streaks" which were fingerprinted are approximately indicated by the bars in Figure 1. Since fingerprint B was poorly reproduced, some homologous peptides in A and B are designated by the same numbers to assist comparison. See the legend to Figure 2 for other details.

microsomes from xenobiotic-treated and untreated rats of five different strains (unpublished observations).

Induction of Cytochromes P-450b$_{LE}$ and P-450e. Cytochromes P-450b$_{LE}$ and P-450e are the only members of a closely related multigene family represented in the colony of Blu-Long-Evans rats used in the present study (Vlasuk et al., 1982). The antibody used in a previous immunoquantitative study (Thomas et al., 1981) cannot distinguish these forms (Ryan et al., 1982a; Vlasuk et al., 1982). However, it was found that the levels of *one* or *both* of these enzymes were markedly increased in hepatic microsomes by phenobarbital, SKF-525A, Aroclor-1254, γ-chlordane, and *trans*-stilbene oxide (Thomas et al., 1981), whereas their induction by isosafrole was not as large.[2] The results in Figure 1 indicate that in all cases both cytochromes P-450b$_{LE}$ and P-450e are induced by these agents and that the levels of cytochrome P-450$_{LE}$ were always ~2 times greater than those for cytochrome P-450e (Figure 1D–H).[3] These results suggest that the inductions of cytochromes P-450b$_{LE}$ and P-450e may be subject to coordinate control.

Induction of Epoxide Hydrolase and Cytochromes P-450a, P-450c, and P-450d. In general, the levels of hepatic microsomal epoxide hydrolase and cytochromes P-450a and P-450c suggested by the results in Figure 1 are consistent with those reported previously from immunoquantitation studies (Thomas et al., 1981).

At the present time, the immunoquantitation of cytochrome P-450d in microsomes has not been reported. The microsomal level of this hemoprotein is significantly increased by all of the xenobiotics tested that induce cytochrome P-450c [i.e., 3-methylcholanthrene, isosafrole, and Aroclor-1254 (see Figure 1B,D,F)]. However, in contrast with the results for cytochromes P-450b$_{LE}$ and P-450e, it appears that the *relative* levels of cytochromes P-450c and P-450d depend on the inducing agent. The level of cytochrome P-450d appears to be much greater vis-ā-vis that of cytochrome P-450c in microsomes from isosafrole-treated rats (Figure 1D), when compared to microsomes from either 3-methylcholanthrene- or Aroclor-1254-treated rats (parts B and F of Figure 1, respectively). Therefore, even though cytochromes P-450c and P-450d share some common immunochemical determinants (Reik et al., 1982), it appears that their induced levels are regulated independently.

Induction of Unidentified Major Microsomal Proteins. Treatment of Blu-Long-Evans rats with phenobarbital not only induced hepatic epoxide hydrolase and cytochromes P-450b$_{LE}$ and P-450e but also significantly increased the levels of polypeptides previously designated PB1 and PB2 which could represent additional forms of cytochrome P-450 (Vlasuk et al., 1982; Vlasuk & Walz, 1982).

Polypeptide PB2 is present in hepatic microsomes from untreated rats (Figure 1A) and is *decreased* by treatment with either 3-methylcholanthrene and possibly Aroclor-1254 (Figure 1B,F). On the other hand, with the exception of Aroclor-1254, all of the xenobiotics which induce cytochromes P-450b$_{LE}$ and P-450e also induce this protein. These results are consistent with those reported previously for Fischer-344 rats (Vlasuk & Walz, 1982) and suggest that inducing agents for cytochromes P-450c and P-450d are antagonistic with those that induce cytochromes P-450b$_{LE}$ and P-450e regarding the level of this polypeptide.

[3] The results for phenobarbital induction were shown previously (Vlasuk et al., 1982).

Table I: Effect of Xenobiotic Treatment on Major Polypeptides in Hepatic Microsomes from Immature, Male Long-Evans Rats[a]

xenobiotic	polypeptide[b] a	b	c	d	e	h	1	2
control	+	0	0	0	0	+	0	+
phenobarbital	I	I	0	0	I	I	I	I
3-methylcholanthrene	I	0	I	I	0	I	0	R
pregnenolone-16α-carbonitrile	+	0	0	0	0	I	I	+
isosafrole	+,I	I	I	I	I	I	I	+
SKF-525A	+,I	I	0	0	I	+	I	I
Aroclor-1254	I	I	I	I	I	I	I	+,R
γ-chlordane	+,I	I	0	0	I	I	0	I
trans-stilbene oxide	+,I	I	0	0	I	I	I	I

[a] Based on the results in Figure 1 and previous studies (Thomas et al., 1981; Vlasuk et al., 1982). [b] Designations are described in Figure 1; (0) none detectable or trace amounts; (+) control level; (I) induced (greater than control level); (R) repressed (less than control level); double entries express uncertainty.

Polypeptide PB1 is not detectable in hepatic microsomes from untreated rats (Figure 1A) but is significantly induced by all of the xenobiotics that increase the levels of cytochromes P-450b_{LE} and P-450e, with the apparent exception of γ-chlordane (Figure 1D–H). Furthermore, this polypeptide was the only microsomal species that detectably increased after treatment of rats with pregnenolone-16α-carbonitrile (Figure 1C).[4] Microsomal polypeptide PB1 migrates in IF/Na-DodSO$_4$ gels with an apparent molecular weight of 51 000 and an apparent p*I* value which is higher than that for all of the other purified rat liver cytochromes P-450. These properties of polypeptide PB1 (i.e., its inducibility by pregnenolone-16α-carbonitrile, size, and relative charge) are consistent with its being cytochrome P-450-PCN which is a unique form of the enzyme purified from pregnenolene-16α-carbonitrile-treated rats (Elshourbagy & Guzelian, 1980). The fingerprint for microsomal polypeptide PB1 induced by pregnenolone-16α-carbonitrile (data not shown) was the same as that reported for its corresponding species from phenobarbital-treated rats (Vlasuk et al., 1982), which verifies that both of these xenobiotics apparently induce the same protein species. The identification of polypeptide PB1 as cytochrome P-450-PCN is further substantiated by recent immunoquantitation studies which indicated that cytochrome P-450-PCN exists at barely detectable levels in hepatic microsomes from control and 3-methylcholanthrene-treated rats but exists at levels of 0.2 and 0.4 nmol/mg of microsomal protein after treatment with phenobarbital and pregnenolone-16α-carbonitrile, respectively (Heuman et al., 1982). If this tentative identification of polypeptide PB1 is correct, then the present results indicate that cytochrome P-450-PCN is also induced by isosafrole, SKF-525A, and Aroclor-1254.

The effect of xenobiotic treatment on major polypeptides associated with hepatic microsomes from sexually immature, male Blu-Long-Evans rats is summarized in Table I. The patterns of induced polypeptides for phenobarbital and *trans*-stilbene oxide are *qualitatively* the same. A qualitative similarity was also apparent for polypeptides induced by Aroclor-1254 and isosafrole. However, all of the other xenobiotics tested yielded unique arrays of major induced polypeptides. If significant *quantitative* differences are also considered, then the results for each xenobiotic treatment appear to be unique. For example, the phenobarbital and *trans*-stilbene oxide induced polypeptide patterns have already been distinguished regarding the levels of cytochromes P-450b_{LE} plus P-450e and epoxide hydrolase (Thomas et al., 1981), whereas those for Aroclor-1254 and isosafrole clearly differ regarding the levels of cytochromes P-450b_{LE}, P-450e, and P-450d (Figure 1D,F).

In every case examined, it has been demonstrated that the inductions of rat liver epoxide hydrolase (Gonzalez & Kasper, 1980; Pickett & Lu, 1981) and several cytochrome P-450 isozymes (Elshourbagy et al., 1981; Bresnick et al., 1981; Adesnick et al., 1981) are associated with increased levels of their corresponding mRNAs. Therefore, it is tempting to conclude that the idiosyncratic microsomal, polypeptide patterns observed in the present study result exclusively from interaction of xenobiotics and/or their metabolites with control elements which regulate transcription/translation processes for the eight, relevant polypeptides. Nevertheless, other factors may influence the observed levels of these microsomal proteins such as (1) tissue concentration and duration of the various inducing agents, (2) specific effects of xenobiotics or their metabolites on the rates of polypeptide turnover, and (3) different time courses for polypeptides approaching their induced steady-state levels (Parkinson et al., 1982). It is also possible that some of the polypeptides listed in Table I are subject to negative control since repression of putative male-specific cytochrome(s) P-450 by phenobarbital and 3-methylcholanthrene has been observed (Vlasuk & Walz, 1982) and decreased levels of noninduced forms of the enzyme after Aroclor-1254 or 3-methylcholanthrene treatment have been reported (Thomas et al., 1981; Parkinson et al., 1982; McIntosh et al., 1980).

Notwithstanding these considerations, it appears that cytochromes P-450b_{LE} and P-450e are coordinately regulated at the level of transcription since mRNAs for these distinct gene products are elevated after phenobarbital treatment (Adesnick et al., 1981; Walz et al., 1982) and virtually the same *relative* proportions of these polypeptides are observed in hepatic microsomes from rats treated with chemically diverse agents (see Figure 1). Furthermore, the hepatic microsomal level of cytochrome P-450b_H [a variant form of cytochrome P-450b_{LE} (Ryan et al., 1982a,b)] was always greater than that for cytochrome P-450e on days 1–4 during the course of phenobarbital treatment of Cr-Holtzman rats (Vlasuk et al., 1982).

The induction specificity for polypeptide PB1 (tentatively identified as cytochrome P-450-PCN; see above) appears to be similar to that for cytochrome P-450b_{LE} and P-450e with respect to Aroclor-1254, phenobarbital, SKF-525A, *trans*-stilbene oxide, and isosafrole (see Figure 1). Nevertheless, the regulation of this gene product is distinguished by its unique responsiveness to pregnenolone-16α-carbonitrile (see Figure 1) and its apparent insensitivity to γ-chlordane, which is a potent inducer of cytochromes P-450b_{LE} and P-450e.

The results of this study provide a more detailed phenomenological basis for understanding the variety of possible induction controls involved in regulating xenobiotic-metabolizing enzymes in rat liver.

[4] Gelcode (Upjohn Diagnostics) silver staining of these IF/NaDodSO$_4$ gels also indicated that polypeptide PB1 was the only induced species.

References

Adesnick, M., Bar-Nun, S., Maschio, F., Zunich, M., Lippman, A., & Bard, E. (1981) *J. Biol. Chem. 256*, 10340–10345.

Bresnick, E., Brosseau, M., Levin, W., Reik, L., Ryan, D. E., & Thomas, P. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4083–4087.

Conney, A. H. (1967) *Pharmacol. Rev. 19*, 317–366.

Elshourbagy, N. A., & Guzelian, P. S. (1980) *J. Biol. Chem. 255*, 1279-1285.
Elshourbagy, N. A., Barwick, J. L., & Guzelian, P. S. (1981) *J. Biol. Chem. 256*, 6060-6068.
Fisher, G. J., Fukushima, H., & Gaylor, J. L. (1981) *J. Biol. Chem. 256*, 4388-4394.
Gibson, G. G., Orton, T. C., & Tamburini, P. P. (1982) *Biochem. J. 203*, 161-168.
Gonzalez, F. J., & Kasaper, C. B. (1980) *Biochem. Biophys. Res. Commun. 93*, 1254-1258.
Guengerich, F. P. (1978) *J. Biol. Chem. 253*, 7931-7939.
Guengerich, F. P., Wang, P., Mitchell, M. B., & Mason, P. S. (1979) *J. Biol. Chem. 254*, 11248-11254.
Heuman, D. M., Gallagher, E. J., Barwick, J. L., Elshourbagy, N. A., & Guzelian, P. W. (1982) *Mol. Pharmacol. 21*, 753-760.
Lu, A. Y. H., & Levin, W. (1974) *Biochim. Biophys. Acta 344*, 205-240.
Lu, A. Y. H., Ryan, D. E., Jerina, D. M., Daly, J. W., & Levin, W. (1975) *J. Biol. Chem. 250*, 8283-8288.
McIntosh, P. R., Peacock, A. C., & Gelboin, H. V. (1980) *Biochim. Biophys. Acta 627*, 290-300.
Parkinson, A., Thomas, P. E., Ryan, D. E., Reik, L. M., Safe, S., Robertson, L. W., Sparling, J., & Levin, W. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1299.
Pickett, C. B., & Lu, A. Y. H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 893-897.
Reik, L. M., Levin, W., Ryan, D. E., & Thomas, P. E. (1982) *J. Biol. Chem. 257*, 3950-3957.
Ryan, D. E., Thomas, P. E., Korzeniowski, D., & Levin, W. (1979) *J. Biol. Chem. 254*, 1365-1374.
Ryan, D. E., Thomas, P. E., & Levin, W. (1980) *J. Biol. Chem. 255*, 7941-7955.
Ryan, D. E., Thomas, P. E., & Levin, W. (1982a) *Arch. Biochem. Biophys. 216*, 272-288.
Ryan, D. E., Thomas, P. E., Wood, A. W., Walz, F. G., Jr., & Levin, W. (1982b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1403.
Thomas, P. E., Reik, L. M., Ryan, D. E., & Levin, W. (1981) *J. Biol. Chem. 256*, 1044-1052.
Vlasuk, G. P., & Walz, F. G., Jr. (1980) *Anal. Biochem. 105*, 112-120.
Vlasuk, G. P., & Walz, F. G., Jr. (1982) *Arch. Biochem. Biophys. 214*, 248-259.
Vlasuk, G. P., Ghrayeb, J., Ryan, D. E., Reik, L., Thomas, P. E., Levin, W., & Walz, F. G., Jr. (1982) *Biochemistry 21*, 789-798.
Walz, F. G., Jr., Vlasuk, G. P., Omiecinski, C., Bresnick, E., Thomas, P. E., Ryan, D. E., & Levin, W. (1982) *J. Biol. Chem. 257*, 4023-4026.

Further Studies on the Binding Characteristics of Rabbit Liver Galactose/*N*-Acetylgalactosamine-Specific Lectin†

Reiko T. Lee, Robert W. Myers, and Yuan Chuan Lee*

ABSTRACT: The affinity of various carbohydrates for the galactose/*N*-acetylgalactosamine-specific lectin of the rabbit liver was assessed by determining the effect of these carbohydrates on the binding of [^{125}I]asialoorosomucoid (^{125}I-ASOR) by the lectin. To obtain the concentration of the inhibitor that causes 50% reduction in the ^{125}I-ASOR binding (I_{50}), we carried out inhibition assays with fixed concentrations of ^{125}I-ASOR and the purified, detergent-solubilized lectin, while the concentrations of the inhibitors were varied. The concentrations of the ^{125}I-ASOR and the lectin were chosen such that the I_{50} value obtained closely approximates the dissociation constant of the inhibitor. Previously, we had shown that equatorial 2-hydroxyl (or acetamido), equatorial 3-hydroxyl, and axial 4-hydroxyl groups of a D-galactopyranosyl (or 2-acetamido-2-deoxy-D-galactopyranosyl) residue in the neoglycoprotein ligand participate in the binding to the lectin [Stowell, C. P., Lee, R. T., & Lee, Y. C. (1980) *Biochemistry 19*, 4904-4908; Lee, R. T. (1982) *Biochemistry 21*, 1045-1050]. In this study, we demonstrate that the methylene group (C-6) and certain aglycons also contribute to the binding. The presence of an unsaturated group such as C=NH at the γ position to the anomeric carbon enhances the binding of an equatorially oriented aglycon. In addition, there seems to be a nonspecific hydrophobic interaction between some aglycons and the lectin binding site. Thus altogether five groups (aglycon, 2-OH or 2-NHAc, 3-OH, 4-OH, and 6-CH_2–) in a galactopyranoside (or *N*-acetylgalactosaminide) have been shown to participate in lectin–ligand interactions. However, not all five groups are absolutely necessary for binding, since significant binding to the liver lectin occurs when only four of these groups are present.

We have previously investigated the ligand structural requirements of the galactose/*N*-acetylgalactosamine-specific lectin of rabbit liver using bovine serum albumin (BSA)[1] derivative containing various galactose[2] analogues as inhibitors of ^{125}I-ASOR binding to the lectin (Krantz et al., 1976; Stowell et al., 1980). These early experiments were performed with the amidino-type BSA neoglycoproteins having Glyc—$SCH_2C(=NH)NH$—protein structure (Lee et al., 1976). It was found that the BSA derivatives of this type containing either glucose or L-arabinose were as inhibitory as galactose-containing BSA derivatives, implying that the lectin requires neither the axial 4-OH nor the –CH_2OH substituent on the C-5. These results suggested that this lectin might have

† From the Department of Biology and the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland 21218. *Received June 14, 1982.* Contribution No. 1179 from the McCollum-Pratt Institute, The Johns Hopkins University. Supported by U.S. Public Health Service–National Institutes of Health Research Grants AM9970 and CA21901.

[1] Abbreviations: BSA, bovine serum albumin; ASOR, asialoorosomucoid; TLC, thin-layer chromatography.

[2] All sugars are of the D configuration unless otherwise specified.

0006-2960/82/0421-6292$01.25/0