Immunological Studies on Two Electrophoretically Homogeneous Forms of Rabbit Liver Microsomal Cytochrome P-450: P-450LM2 and P-450LM4

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Two electrophoretically homogeneous forms of liver microsomal cytochrome P-450 (P-450LM), designated P-450LM2 and P-450LM4, by their relative mobilities, were previously isolated from rats induced with phenobarbital and 7β-naphthoflavone, respectively. In the present immunological study, rabbits and goats were inoculated with these purified proteins. Rabbits produced antibodies against P-450LM, but not against P-450LM4, whereas goats produced antibodies against both cytochromes. Antibodies against P-450LM were purified from anti-LM serum by preparation of the P-450LM-antibody precipitate. The ability of Fab' fragments prepared from the purified antibodies to bind to P-450LM was studied by fluorescence quenching, and it was found that about three Fab' fragments from rabbit and ten from goat were bound per molecule of cytochrome.

In Ouchterlony double diffusion studies, all antisera yielded only one precipitin band with both purified and crude preparations of the corresponding antigen, thereby providing additional evidence for the homogeneity of the cytochromes. No cross-reactions observable by precipitin band formation were detected between anti-LM sera and P-450LM, or between anti-LM serum and P-450LM4. Competitive binding studies with radiolabeled cytochromes confirmed that rabbit anti-LM does not cross-react with P-450LM; however, slight but significant cross-reactions were detected by this technique between goat anti-LM and P-450LM, and between goat anti-LM, and P-450LM4. These results indicate that the two cytochromes have significant structural differences.

Benzphetamine binding, P-450LM reduction by NADPH in the presence of the reductase, and benzphetamine hydroxylation were all strongly inhibited by goat anti-LM, Fab' fragments in a reconstituted enzyme system containing P-450LM, whereas in intact microsomes benzphetamine binding was inhibited much less than the other two activities.

These results suggest that the substrate binding site on P-450LM may be embedded in the membrane, while the site of reductase binding or electron transfer is exposed on the microsomal surface.

The cytochrome P-450-containing enzyme system of liver microsomes has been implicated in a wide variety of substrate hydroxylations. The unusually broad substrate specificity is explained, at least in part, by the occurrence of multiple forms of the cytochrome. This laboratory has reported the purification to electrophoretic homogeneity of the phenobarbital inducible form, P-450LM, and the β-naphthoflavone-inducible form, P-450LM4, as well as the partial purification of other forms from rabbit liver microsomes (3-6). The isolation of the cytochrome from 3-methylcholanthrene-treated rats and rabbits and from phenobarbital-treated rats has been described by investigators at Hoffmann-La Roche (7, 8), and from phenobarbital- and 3-methylcholanthrene-treated rabbits by investigators in Japan (9, 10).

In the studies described in the present paper, antibodies elicited against highly purified P-450LM and LM, were used to assess the immunological relationships between the two cytochromes and to study the orientation of P-450LM in the microsomal membrane. Welton et al. (11) have used several immunological methods to show that antibody prepared against protease-treated cytochrome P-420 obtained from rat liver microsomes does not cross-react with two higher molecular weight hemeproteins, and Thomas et al. (12) have demonstrated that antibody prepared against purified hepatic cytochrome P-450 from 3-methylcholanthrene-treated rats cross-reacts poorly with the purified cytochrome from phenobarbital-treated rats.2

1 The abbreviations used are: P-450LM, liver microsomal cytochrome P-450; dilauroyl-GPC, dilauroylglyceryl-3-phosphorylcholine; anti-LM, and anti-LM4, antibodies elicited against P-450LM and P-450LM4, respectively.

2 The experimental methods are presented in a miniprint format immediately following this paper on p. 3261. For the convenience of those who prefer to obtain these methods in the form of 4 pages of full-size photocopies, it is available as JBC Document No. 76M-1236. Orders should specify the title, authors, and reference to this paper, the JBC Document Number, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $1.00 per set of photocopies.
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Production of Antiserum—Antibodies were produced in rabbits against P-450LM2; at equivalence, an average of 1.4 nmol of P-450LM were bound/ml of antiserum. In contrast, no antibody against P-450LM could be detected in the serum of rabbits inoculated with P-450LM. Antibodies against both cytochromes were produced in goats in relatively high concentrations; at equivalence, anti-LM2 serum bound 15.1 nmol of P-450LM2/ml, and anti-LM1 serum bound 12.3 nmol of P-450LM1/ml. The three antisera produced normal precipitin curves in quantitative studies, an example of which is shown in Fig. 1.

Properties of Purified Antibodies and Fab' Fragments—Antibodies specific for P-450LM2, purified from rabbit and goat anti-LM2 sera, yielded precipitin curves that were highly similar to those obtained with crude antisera. For reasons that are not clear, the binding of the cytochrome to the purified antibodies was about twice as great when small amounts of preimmune y-globulin purified by DEAE-cellulose column chromatography or for the specific anti-LM1 preparations obtained from the antigen-antibody precipitate. The molecular weights, estimated in calibrated gels, were 50,000 and 25,000, corresponding to the y-globulin heavy and light chains, respectively.5

Fab' fragments prepared from preimmune y-globulin and from the specific antibody preparations were also treated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol (4, 34) revealed only two protein bands for pre-immune y-globulin purified by DEAE-cellulose column chromatography or for the specific anti-LM1 preparations obtained from the antigen-antibody precipitate. The molecular weights, estimated in calibrated gels, were 50,000 and 25,000, corresponding to the y-globulin heavy and light chains, respectively.5

Fab' fragments prepared from preimmune y-globulin and from the specific antibody preparations were also treated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and both exhibited only one protein band with a mobility corresponding to a molecular weight of 25,000. Experiments employing P-450LM2-saturated CM-cellulose, described in detail in the miniprint supplement, showed that at least 95% of the anti-LM2 Fab' fragments from both goat and rabbit were bound to P-450LM2.

The binding of P-450LM2 to anti-LM2 Fab' fragments was accompanied by quenching of the fluorescence at 325 nm, as shown in Fig. 2. At saturation, this cytochrome quenched the fluorescence of rabbit and goat Fab' fragments by 20% and 18%, respectively. The intersection of the line indicating maximum fluorescence quenched with the initial slope of the plot occurs at a molar antigen/antibody molar ratio of 0.3 for rabbit and 0.1 for goat Fab' fragments. These results show that approximately three rabbit Fab' fragments and about ten goat Fab' fragments are bound per P-450LM2 at saturation. It has been shown by others that five Fab' fragments are bound per molecule of cytochrome c (36) and 12 per molecule of hemoglobin (27), which have molecular weights of 12,500 and 62,000, respectively. For comparison, the molecular weight of P-450LM2 is 48,500 (6).

Ouchterlony Double Diffusion Analysis—The specificity of the antisera was evaluated by Ouchterlony double diffusion analysis. As shown in Fig. 3A, rabbit anti-LM2 formed a single precipitin band with P-450LM2 or with phenobarbital-induced microsomes solubilized with 0.5% deoxycholate. No bands were seen, however, with 3-fold greater levels of P-450LM1, P-450LM1,2 (a mixture of LM1 and LM2) (5), deoxycholate-solubilized β-naphthoflavone-induced microsomes, or deoxycholate solubilized control microsomes. Trace amounts of P-450LM2 are apparently present in control microsomes from rabbits, since the precipitin band from P-450LM2 does not extend into the well containing control microsomes.2 Exoxycholate was included in the agar gel in Experiment A, but essentially the same results were obtained when the detergent was omitted from the agar; therefore, it was omitted in further experiments. In contrast to the results presented here with uninduced rabbit liver microsomes, Welton et al. (11) reported that the phenobarbital-inducible cytochrome P-450 from rat liver is present in significant amounts in control microsomes as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunodiffusion, thus indicating a quantitative difference in these two species. Fig. 3B shows that goat anti-LM1 serum formed a single precipitin band with P-450LM1 or with a relatively crude fraction from phenobarbital-induced microsomes, but no band was formed with a 5-fold greater concentration of P-450LM1. Similarly, in Fig. 3C, goat anti-LM4 formed a single band with P-450LM4, or a relatively crude fraction from β-naphthoflavone-induced microsomes, but did not cross-react with a 5-fold greater concentration of P-450LM4. These results demonstrate the immunochemical dissimilarity of P-450LM2 and LM1 and provide additional evidence for the homogeneity of these cytochromes.

Radioimmune Assays—In addition to the studies already described, a more sensitive radioimmune assay based on competitive binding was employed. A series of experiments were

2 The presence of trace amounts of P-450LM2 in control microsomes has been confirmed by its partial purification during the isolation of P-450LM1, from this source (S. B. Dahl and M. J. Coon, unpublished data).

Fig. 1. Quantitative precipitin assay for goat anti-LM2 serum with highly purified P-450LM2. The reaction mixtures were as described in the text and contained 0.02 ml of serum.

Fig. 2. Assay of rabbit and goat anti-LM1 Fab' fragments by fluorescence quenching. Increasing amounts of P-450LM1 were added to a 1.0 μM solution of Fab' fragments. The curves have been extrapolated (---) to give the binding stoichiometry.
FIG. 3. Ouchterlony double diffusion analysis of antisera. Experiment A, the agar contained 0.5% deoxycholate. The center well contained rabbit anti-LMγ-globulin purified by DEAE-cellulose chromatography (1.5 mg of protein). The amounts of P-450LM and total protein, respectively, in the other wells were as follows: Well 1, phenobarbital-induced microsomes solubilized with 0.5% deoxycholate (0.3 nmol, 0.08 mg); Well 2, P-450LM, (0.3 nmol, 0.02 mg); Well 3, P-450LM, (0.9 nmol, 0.20 mg); Well 4, P-450LM, (0.9 nmol, 0.07 mg); Well 5, P-450LM, (0.9 nmol, 0.39 mg); and Well 6, control (uninduced) microsomes solubilized with 0.5% deoxycholate (0.9 nmol, 0.47 mg). Experiment B, the agar contained no deoxycholate. The center well contained 0.075 ml of goat anti-LMγ serum. Well 1, P-450LM, (0.75 nmol, 0.04 mg); Well 2, phenobarbital-induced microsomes, 11 to 13% polyethylene glycol fraction (0.75 nmol, 0.11 mg); Well 3, phenobarbital-induced microsomes, 11 to 13% polyethylene glycol fraction (3.75 nmol, 0.54 mg); Well 4, P-450LM, (0.75 nmol, 0.04 mg); Well 5, P-450LM, (3.75 nmol, 0.22 mg); and Well 6, 0.075 ml of 0.02 M phosphate buffer, pH 7.4. Experiment C, the agar contained no deoxycholate. The center well contained 0.04 ml of goat anti-LMγ serum and 0.035 ml of phosphate buffer. Well 1, P-450LM, (0.75 nmol, 0.04 mg); Well 2, β-naphthoflavone-induced microsomes, 8 to 12% polyethylene glycol fraction (0.75 nmol, 0.12 mg); Well 3, β-naphthoflavone-induced microsomes, 8 to 12% polyethylene glycol fraction (3.75 nmol, 0.62 mg); Well 4, P-450LM, (0.75 nmol, 0.04 mg); Well 5, P-450LM, (3.75 nmol, 0.21 mg); and Well 6, 0.075 ml of phosphate buffer.

FIG. 4. Competition between 125I-labeled and unlabeled cytochromes for antisera. Experiment A, increasing amounts of unlabeled cytochromes, as indicated, were incubated in a reaction mixture containing 0.01 ml of rabbit anti-LMγ serum. 125I-labeled P-450LM, (12.5 pmol, 0.76 μg) was added, and the inhibition of binding of the labeled cytochrome was determined. Experiment B, conditions were identical with those in Experiment A, except that 84 pmol (5.1 μg) of 125I-labeled P-450LM, and 0.015 ml of goat anti-LMγ serum were used. Experiment C, conditions were the same, except that 0.65 pmol (0.04 μg) of 125I-labeled P-450LM, and 0.001 ml of goat anti-LMγ serum were used. The specific contents of unlabeled antigens were 16.8 nmol/mg for P-450LM, and 16.9 nmol/mg for P-450LM,. Maximum binding of labeled cytochrome (i.e. no inhibition) was defined as the radioactivity precipitated when no unlabeled cytochrome was added.

Inhibition of Benzphetamine N-Demethylation – The effects of goat anti-LMγ-globulin and Fab’ fragments on the benzphetamine-dependent oxidation of NADPH in the reconstituted system are shown in Fig. 5A. At high levels both prepa-
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Pre-immune Fab Fragments

**Fig. 5.** Inhibition of benzphetamine N-demethylation by goat anti-LM γ-globulin and Fab' fragments. The data presented take into account the presence of two sites per molecule of γ-globulin and one per molecule of Fab' fragments. Experiment A, various amounts of goat anti-LM γ-globulin or Fab' fragments were incubated with the reconstituted system containing 0.1 nmol of P-450LM, for 30 min at 30°C prior to the addition of NADPH. Activity was measured by the disappearance of absorbance at 340 nm. Experiment B, various amounts of the antibodies were incubated with phenobarbital-induced microsomes (containing 0.3 nmol of P-450LM), and the formation of radioactive formaldehyde from labeled benzphetamine was determined.

Inhibition of benzphetamine N-demethylation by goat anti-LM γ-globulin and Fab' fragments was shown in Fig. 5. The data presented take into account the presence of two sites per molecule of γ-globulin and one per molecule of Fab' fragments. Experiment A, various amounts of goat anti-LM γ-globulin or Fab' fragments were incubated with the reconstituted system containing 0.1 nmol of P-450LM, for 30 min at 30°C prior to the addition of NADPH. Activity was measured by the disappearance of absorbance at 340 nm. Experiment B, various amounts of the antibodies were incubated with phenobarbital-induced microsomes (containing 0.3 nmol of P-450LM), and the formation of radioactive formaldehyde from labeled benzphetamine was determined.

Inhibition of N-demethylation activity as judged by formaldehyde formation in phenobarbital-induced microsomes is shown in Fig. 5B. After correction for the number of binding sites per antibody molecule, the effects of γ-globulin and Fab' fragments were nearly identical, and the maximum inhibition was about 80%. The noninhibitable benzphetamine demethylation activity in microsomes is probably due to the presence of forms of the cytochrome other than P-450LM, which are not strongly bound by the antibodies. For example, Haugen et al. (5) demonstrated that P-450LM, and a mixture of P-450LM, and LM1 had slight but significant activity with benzphetamine.

**Inhibition of Benzphetamine Binding**—Fig. 6A shows that goat anti-LM Fab' fragments strongly inhibited the binding of benzphetamine to P-450LM, in the reconstituted system. At a molar ratio of 8.4 Fab' fragments per P-450LM, approximately 80% inhibition of binding occurred, as determined by the estimated magnitude of the difference spectrum at infinite benzphetamine concentration. In contrast to the results observed with purified P-450LM, Fab' fragments had little effect on benzphetamine binding to phenobarbital-induced microsomes, as shown in Fig. 6B.

**Fig. 6.** Effect of goat anti-LM Fab' fragments on benzphetamine binding. The complete reaction mixtures were incubated for 5 min at 22°C before the spectra were recorded. Experiment A, varying amounts of benzphetamine were added to P-450LM (0.5 nmol, 0.03 mg) in 1.0 ml of 0.1 M phosphate buffer, pH 7.4, containing 30 μg of dilauroyl-GPC. In the presence of Fab' fragments at the concentrations indicated. Experiment B, varying amounts of benzphetamine were added to phenobarbital-induced microsomes (0.5 nmol, 0.13 mg of protein) in 1.0 ml of 0.1 M phosphate buffer, pH 7.4, in the presence of Fab' fragments at the concentrations indicated.

**Fig. 7.** Effect of Fab' fragments on the reduction of cytochrome P-450 by NADPH. Experiment A, varying amounts of goat anti-LM Fab' fragments were added to the reconstituted system used for reduction of P-450LM, as described in the miniprint supplement. Experiment B, goat anti-LM Fab' fragments were added to a reaction mixture containing phenobarbital-induced microsomes (2.0 nmol of P-450LM, 0.53 mg of protein). In both experiments, the Fab' fragments were present during a 5-min preincubation prior to the addition of NADPH.

**Inhibition of Reduction of P-450**—Coon et al. (40) observed earlier that the fast phase of reduction of partially purified P-450LM by NADPH in the reconstituted system was complete in 3 s, while the remainder of the cytochrome was reduced in a slow phase taking several minutes. The effects of goat anti-LM Fab' fragments on the reduction of purified P-450LM by NADPH are shown in Fig. 7A. Fab' fragments inhibited both the amount of cytochrome reduced during the fast phase and the final extent of reduction. In experiments not shown, dithionite reduction of P-450LM was not affected by Fab' fragments. The results of similar experiments carried out with phenobarbital-induced rabbit liver microsomes are shown in Fig. 7B. Again, a fast and a slow phase of reduction were present, as reported earlier by Gigon et al. for rat liver micro-
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Fig. 8. Comparison of the effects of anti-LM, Fab' fragments on N-demethylation of benzphetamine, benzphetamine binding, and the extent of P-450LM, reduction in the reconstituted system (A) and in phenobarbital-induced microsomes (B).

DISCUSSION

The immunochemical studies reported in this paper on two forms of cytochrome P-450 purified from rabbit liver microsomes show that antibodies are produced in rabbits against P-450LM and, in goats against both P-450LM and LM. Rabbit anti-LM is quite specific for P-450LM, as shown by the lack of cross-reaction with P-450LM, in Ouchterlony diffusion and radioimmuno competitive binding studies. Goat anti-LM, however, shows a weak cross-reaction with P-450LM. This species difference may be a consequence of the lower number of binding sites on P-450LM recognized by rabbit antibodies as compared with goat antibodies, as determined by fluorescence quenching. Goat anti-LM shows a weak cross-reaction with P-450LM, thereby further indicating that the two cytochromes have only minor immunochemical determinants in common. Thus, there appear to be significant differences in the tertiary structures of these two cytochromes from liver microsomes. In contrast, P-450LM and bacterial P450cam (38) show extensive immunological cross-reactivity (37, 39) even though they differ markedly in substrate specificity and in the requirement of the microsomal hemoprotein for a phospholipid and the bacterial hemoprotein for an iron-sulfur protein for hydroxylation activity.

P-450LM, and LM, isolated from phenobarbital- and p-naphthoflavone-induced animals, respectively, appear to be homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by determination of the COOH-terminal amino acid residues; carboxypeptidase treatment gave arginine from P-450LM, and lysine from P-450LM, in close to quantitative yields (6). The Ouchterlony studies described in the present report provide further evidence for the homogeneity of these cytochromes. Evidence has also been obtained that P-450LM, isolated from control, phenobarbital-induced, or p-naphthoflavone-induced rabbit liver microsomes is the same protein (6). In experiments not given in the present paper, electrophoretically homogeneous P-450LM, from these three sources was examined by the Ouchterlony technique, using antisera to the p-naphthoflavone-induced preparation of the cytochrome, and the three exhibited complete identity. The possibility should be considered, however, that highly purified P-450LM, and P-450LM, may each contain other proteins so similar that they are not distinguished by any of the techniques so far employed.

Welton et al. (11) prepared a protease-treated cytochrome P-420 hemeprotein in electrophoretically homogeneous form from phenobarbital-treated rats. This protein, which gave a single band by Ouchterlony double diffusion analysis, did not cross-react with hemeproteins of higher molecular weight in solubilized microsomes. Immunochemical studies by Thomas et al. (12) on highly purified cytochrome P-450 from phenobarbital- and 3-methylcholanthrene-treated rats showed that these cytochromes are immunochemically different and indicated the presence of at least three hemeproteins in the enzyme preparation from phenobarbital-induced animals. In the reconstituted rabbit liver system containing P-450LM, anti-LM, Fab' fragments strongly inhibit benzphetamine binding, cytochrome reduction by NADPH in the presence of the reductase, and benzphetamine hydroxylation (demethylation), whereas in intact microsomes benzphetamine binding is inhibited much less than the other two activities. These results suggest that the substrate binding site of the cytochrome may be embedded in the membrane, while the site of reductase binding or electron transfer is exposed on the microsomal surface. The finding of others (17, 45-47) that the flavin-containing portion of NADPH-cytochrome P-450 reductase is released by proteases in the absence of detergents indicates that it is on the exterior of the microsomal membrane and supports the view that this is the location of electron transfer to the cytochrome.

Acknowledgments—We are grateful to Dr. Karl M. Dus, Department of Biochemistry, St. Louis University, for helpful discussions, to Sylvia B. Dahl for purifying P-450LM, and LM, and to Barbara M. Michniewicz for purifying NADPH-cytochrome P-450 reductase.

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EXPERIMENTAL METHODS

Purification of Microsomal Enzyme - Liver microsomes were prepared as described previously (3) from rabbits treated with 2,4-BP or KI. Microsomes were subjected to the following purification procedure.

1. Release of microsomal protein by sonication. The procedure of Fiskerblad et al. (4) was used with the following modifications: (a) the duration of sonication was increased to 2 min; (b) the sonicator was operated at the highest power setting; (c) the solution was centrifuged at 100,000 x g for 1 hr and the supernatant was used.

2. Fractionation of microsomal proteins by polyacrylamide gel electrophoresis. The procedure of O'Farrell et al. (5) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 16 hr; (c) the gel was stained with Coomassie blue.

3. Gel electrophoresis of microsomal proteins by SDS-PAGE. The procedure of Laemmli (6) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

4. Gel electrophoresis of microsomal proteins by isoelectric focusing. The procedure of O'Farrell et al. (5) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

5. Gel electrophoresis of microsomal proteins by SDS-PAGE. The procedure of Laemmli (6) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

6. Gel electrophoresis of microsomal proteins by isoelectric focusing. The procedure of O'Farrell et al. (5) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

7. Gel electrophoresis of microsomal proteins by SDS-PAGE. The procedure of Laemmli (6) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

8. Gel electrophoresis of microsomal proteins by isoelectric focusing. The procedure of O'Farrell et al. (5) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

9. Gel electrophoresis of microsomal proteins by SDS-PAGE. The procedure of Laemmli (6) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

10. Gel electrophoresis of microsomal proteins by isoelectric focusing. The procedure of O'Farrell et al. (5) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

11. Gel electrophoresis of microsomal proteins by SDS-PAGE. The procedure of Laemmli (6) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

12. Gel electrophoresis of microsomal proteins by isoelectric focusing. The procedure of O'Farrell et al. (5) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

13. Gel electrophoresis of microsomal proteins by SDS-PAGE. The procedure of Laemmli (6) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

14. Gel electrophoresis of microsomal proteins by isoelectric focusing. The procedure of O'Farrell et al. (5) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

15. Gel electrophoresis of microsomal proteins by SDS-PAGE. The procedure of Laemmli (6) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

16. Gel electrophoresis of microsomal proteins by isoelectric focusing. The procedure of O'Farrell et al. (5) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

17. Gel electrophoresis of microsomal proteins by SDS-PAGE. The procedure of Laemmli (6) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.

18. Gel electrophoresis of microsomal proteins by isoelectric focusing. The procedure of O'Farrell et al. (5) was used with the following modifications: (a) the gel was run at a constant current of 10 mA per lane; (b) the gel was run for 1 hr; (c) the gel was stained with Coomassie blue.
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