Effects of Phenobarbital, 3-Methylcholanthrene, and Hematin on the Synthesis of Protein Components of Rat Liver Microsomal Membranes*

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SUMMARY

Smooth endoplasmic reticulum was solubilized in sodium dodecyl sulfate and the protein components were fractionated by acrylamide gel electrophoresis. The relative rates of amino acid incorporation into the fractionated protein bands following various drug treatments were measured. The drugs used were phenobarbital and 3-methylcholanthrene, representative of two classes of drugs which induce a variety of microsomal drug-metabolizing activities, and hematin, which causes a decrease in the activities of enzymes associated with drug metabolism.

Each of the three compounds produced distinctive changes in amino acid incorporation into the protein components of smooth endoplasmic reticulum. In each case the greatest change in amino acid incorporation was in a major protein band in the 50,000 molecular weight region of the gel. Cytochrome P-420, the solubilized form of cytochrome P-450, was purified and shown to migrate as a protein with a molecular weight of 50,000, indicating that this protein is contained in the 50,000 molecular weight band. Amino acid incorporation into isolated cytochrome P-420 showed that this protein is responsible for at least part of the drug-mediated changes in amino acid incorporation observed for the 50,000 molecular weight protein band.

Liver endoplasmic reticulum has been the subject of various studies of membrane synthesis and degradation (1-5). This system offers some advantage in studying membrane biogenesis in that the amount of endoplasmic reticulum and the activities of enzymes involved in the metabolism of steroids and various drugs are responsive to changes in hormonal levels (6) and to many xenobiotics (6-8). The principle identified components of the mixed function oxidase system, an electron transport pathway leading to the oxidation of steroids and drugs, are NADPH cytochrome c reductase and cytochrome P-450 (6, 9). The levels of both of these enzymes are responsive to drug treatments (1, 10). Of these, NADPH cytochrome c reductase has been purified and its rate of synthesis shown to be selectively increased in response to administration of phenobarbital (1, 2, 11). However, cytochrome P-450, the terminal oxidase (9) and substrate-binding site (12) of the mixed function oxidase system, has not been amenable to purification from liver microsomes. Consequently, changes in its rate of synthesis, or degradation in response to various drug treatments, or both, have not been studied. Such a study should aid in answering the following questions. (a) Are synthesis and degradation of the mixed function oxidase components coordinately controlled? (b) Is the synthesis of cytochrome P-450, and perhaps other mixed function oxidase components, regulated independently with respect to other membrane proteins? (c) What is the mechanism (or mechanisms) by which membrane proteins are selectively inserted into or removed from previously existing membrane?

The question of whether the synthesis and degradation of cytochrome P-450 is regulated independently with respect to other membrane proteins was considered in an earlier study in which the relative turnover rates of microsomal proteins were measured in the steady state (3). As displayed on SDS acrylamide gels, larger proteins were found to have greater degradation rates. A conspicuous exception to this general correlation was a major protein band in the 50,000 molecular weight region. This band showed a high turnover rate, and several lines of evidence suggested that this band, or a component of it, was cytochrome P-450.

In the present study we have characterized further this protein band under conditions in which cytochrome P-450 content and drug-metabolizing capacities of microsomes are altered by various drug treatments. These experiments were performed both to support the identification of this band as containing cytochrome P-450 and to investigate drug-mediated effects on the rate of its synthesis relative to other microsomal membrane proteins.

METHODS

Isolation of SER—All experiments were performed on livers from male, white Sprague-Dawley rats weighing between 110 to 140 g each. The animals were fasted 18 hours before killing. Livers were perfused through the spleen with 10 ml of cold 0.25 M
sucrose before excision, and homogenized in 4 volumes of 0.25 M sucrose with a glass homogenizer and Teflon pestle. SER was isolated by the method of Dallner (19), modified by use of the SB-253 rotor of an International model B-60 ultracentrifuge. The pelleted SER was suspended by homogenization in 0.5 M KCl, 0.1 M potassium phosphate buffer, pH 7.5, at a concentration of about 10 mg per ml. Part of the fraction was used in measuring enzyme activities; the remainder was prepared for gel electrophoresis as described below.

Enzyme Assays—Cytochrome P-450 was assayed by the method of Lu et al. (14). Carbon monoxide difference spectra of reduced microsomal preparations were measured with a Zeiss spectrophotometer. The solubilized form of this protein, cytochrome P-420, was assayed by its CO difference spectrum at 420 nm. NADPH cytochrome c reductase was also assayed as described by Lu et al. (14).

Microsomal amionopyrine demethylase activity was determined with a modification of the method of Coney et al. (7). Approximately 2 mg of microsomes were added to an incubation mixture containing 0.5 ml of potassium phosphate buffer, pH 7.5, 0.1 ml of 1 M MgCl₂, 0.1 ml of 1 M KCl, 1 ml of 1 mM aminopyrine, and 20 μl of 50 mM NADPH. Distilled water was added to give a final volume of 3 ml. The mixture was incubated at 37°C for 30 min, during which time 20 μl of 30 mM NADPH were added every 5 min. The amount of formaldehyde formed was measured by the method of Nash (15). The ability of microsomes to metabolize zoxazolamine was assayed similarly, using 1 ml of 0.6 mM zoxazolamine in the incubation mixture rather than aminopyrine. The disappearance of zoxazolamine was determined spectrophotometrically (16). Both of these assays were found to be linear with time and enzyme concentration within the ranges used in these studies.

Drug and Isotope Administration—[U-¹⁴C]Leucine (300 mCi per mm) and [4, 5-³H]leucine (2000 mCi per mm) were obtained from Schwarz BioResearch. The isotope was diluted to 1.5 ml in 10 mM NaCl and injected intraperitoneally in two 0.75-ml doses. Sodium phenobarbital (Merck) was dissolved in water and injected intraperitoneally, 100 mg per kg; 3-methylcholanthrene (Calbiochem) was dissolved in corn oil and injected intraperitoneally, 25 mg per kg; heparin (Calbiochem), dissolved in 10 mM KOH, was given intraperitoneally at a dose of 16 mg per kg.

In the experiments designed to measure the relative rates of protein synthesis following drug administration, an experimental animal was given the drug several hours before killing and at the same time a control animal received an equal volume of the drug vehicle (see figure legends for details). Four hours before killing, the experimental animal was given 250 μCi of [³H]leucine and the control, 75 μCi of [¹⁴C]leucine. The 4-hour period between isotope injection and killing allowed for the passage of the rapidly labeled serum proteins through the endoplasmic reticulum before isolation of the SER fraction (2).

SDS Gel Electrophoresis—Homologates of ¹³C and ¹⁴C-labeled SER isolated from equal volumes of liver homogenate and prepared as described above, were combined, sonicated for 20 sec with a Branson sonifier at a power setting of 1, and centrifuged at 105,000 x g for 1 hour to remove loosely bound proteins. The pellet was resuspended by sonication in distilled water and centrifuged at 105,000 x g for 1 hour. This washed SER was then resuspended in 1% TX (v/v) in water to a final protein concentration of about 5 mg per ml, sonicated for 20 sec, and centrifuged at 105,000 x g for 1 hour. The supernatant, designated TX-soluble SER, contained approximately 50% of the washed microsomal protein. This fraction was made 0.1% in SDS (w/v), sonicated for 20 sec, and applied to the gels. The membrane fraction insoluble in 1% TX required 2 to 3 min of sonication in 0.5% SDS for solubilization. It was not necessary to reduce the SDS concentration of this material before electrophoresis (3).

Protein separation by acrylamide gel electrophoresis was carried out on discontinuous gradient acrylamide gels in the presence of 0.1% SDS, as described previously (3). The acrylamide concentration gradients employed in fractionating the TX-soluble and TX-insoluble SER proteins are shown alongside the gels in Fig. 1. Gels, 5 x 75 mm, were used to display the protein patterns (analytical gels); large (preparative) gels, 19 x 75 mm, were employed for radioactive samples, since such gels were capable of resolving an amount of protein (about 5 mg) required to obtain sufficient radioactivity for accurate counting. Approximately 225 μg of protein in 50 to 100 μl were applied to the analytical gels and about 5 mg to the preparative gels. Small amounts of sucrose and tracking dye (bromphenol blue) were included in the sample. Electrophoresis was carried out at a current which did not exceed 0.5 mA per small tube (7.5 mA per large tube). The small gels were stained with 1% (w/v) Fast Green (Eastman) in 10% acetic acid and destained in 10% acetic acid. The large gels were frozen on Dry Ice and stored at -18°C until sliced.

The method for slicing the gel and preparing the slices for scintillation counting has been described previously (3). The gels were sliced into 80 to 90 1-mm slices and the ¹³C and ¹⁴C counts determined for each slice and plotted on a linear scale. The small, stained gels were scanned at 600 nm in a Gilford model 2000 spectrophotometer equipped with a gel-scanning attachment. The radioactivity was matched to the optical density in two ways, both of which take account of the fact that both sets of data are plotted on a linear scale. By one technique RF values were calculated. Alternatively this was done by matching the point of maximum ¹³C counts to the midpoint of the 50,000 molecular weight band (see Fig. 3). Once the correspondence between the two graphs was established, the absorbance of the radioactivity graph was adjusted to match that of the optical density scan, and the ¹³C:¹⁴C ratios were plotted on this scale (see Figs. 4 to 7). In all cases, both methods resulted in identical matching of the two sets of data.

Purification of Cytochrome P-420—The purification of cytochrome P-420, the solubilized form of P-450, was performed by a modification of the methods of Omura and Sato (17). SER, prepared as described above, was homogenized in 0.1 M potassium phosphate buffer, pH 7.5, and centrifuged for 1 hour at 105,000 x g. This and subsequent homogenizations were performed in a glass homogenizer fitted with a Teflon pestle. The pelleted SER was washed once with distilled water and resuspended in 1% TX to a final protein concentration of about 5 mg per ml, then centrifuged at 105,000 x g for 1 hour. The resulting pellet was homogenized in 10 mM Tris-acetate buffer, pH 9.0, at a protein concentration of about 10 mg per ml. The snake venom of Trimeresurus flavoviridis (Sigma), heat treated as described by Imai and Sato (18), was added to a concentration of 0.1% and the mixture was incubated at 4°C under nitrogen atmosphere for 24
hours, followed by overnight dialysis against 10 mM Tris-acetate, pH 10.0. The clear reddish supernatant resulting from centrifugation at 105,000 × g for 1 hour was fractionated further by gel electrophoresis, with preparative scale (18 × 45 mm) discontinuous gradient gels. The gel buffer employed was 20 mM Tris-acetate, pH 10.0. The acrylamide stock solutions for final acrylamide concentrations of 10, 7.5, and 4% were prepared as described previously (3). The gel solutions consisted of 10 ml of gel buffer solution, 5 ml of acrylamide stock, 5 ml of freshly prepared 0.28% ammonium persulfate, and 20 μl of TEMED. The three gel solutions, 7 ml of 10%, and 3 ml each of 7.5 and 4% acrylamide, were layered in a 1-nm inside diameter glass tube, one over the other, before polymerization occurred. The sample, containing about 10 mg of protein and a small amount of sucrose and tracking dye (bromphenol blue) was applied to the gel and electrophoretically treated in 50 mM Tris-acetate buffer, pH 10.0. Electrophoresis was carried out at a current of 5 mA per large tube. The reddish orange band which entered the 10% acrylamide region migrated closely behind the tracking dye. When the tracking dye had run off the bottom of the tube, the cytochrome band was electrophoretically treated into a small buffer chamber which was separated from the lower buffer by a dialysis membrane. The sample was concentrated by lyophilization and stored at −18°C. The yield from 25 mg of SER was about 1.5 mg of the cytochrome P-420 fraction.

The radioactivity of labeled SER and purified cytochrome P-420 was determined by precipitating an aliquot of the sample, containing about 10 mg of protein and a small amount of sucrose and tracking dye (bromphenol blue) was applied to the gel and electrophoretically treated in 50 mM Tris-acetate buffer, pH 10.0. Electrophoresis was carried out at a current of 5 mA per large tube. The reddish orange band which entered the 10% acrylamide region migrated closely behind the tracking dye. When the tracking dye had run off the bottom of the tube, the cytochrome band was electrophoretically treated into a small buffer chamber which was separated from the lower buffer by a dialysis membrane. The sample was concentrated by lyophilization and stored at −18°C. The yield from 25 mg of SER was about 1.5 mg of the cytochrome P-420 fraction.

The drug indicated was administered to three animals and an equal volume of the drug vehicle was given to three control animals several hours before killing. The SER fraction from each animal was isolated and assayed for specific activities. The average specific activity of the three control animals was set at 100%, and the average specific activity of the three experimental animals expressed relative to 100%. Phenobarbital, dissolved in water, was given at a dose of 100 mg per kg 16 hours prior to killing. 3-Methylcholanthrene was dissolved in corn oil and given at a dose 25 mg per kg 16 hours prior to killing. Hematin was dissolved in 10 mM KOH and 10 mg per kg were administered 18, 16, and 4 hours prior to killing. In the phenobarbital plus hematin treatment, the experimental animals received phenobarbital (100 mg per kg) 16 hours, and hematin (16 mg per kg) 18, 16, and 4 hours prior to killing. The drug-induced changes in drug metabolism activities shown here are representative of those observed in the double isotope experiments (Figs. 4 to 7).

### Table I

<table>
<thead>
<tr>
<th>Drug administered</th>
<th>Cytochrome P-420</th>
<th>NADPH cytochrome c reductase</th>
<th>Aminopyrine demethylase</th>
<th>Oxazolamine hydroxylase</th>
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<td>100</td>
<td>185</td>
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<td>3-Methylcholanthrene</td>
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<td>Hematin</td>
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<td>Hematin + phenobarbital</td>
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<td>125</td>
<td>129</td>
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</table>

**FIG. 1.** Gel electrophoresis patterns of total SER, TX-soluble, and TX-insoluble SER subfractions. Fractionation of SER on the basis of solubility in 1% TX as outlined under "Methods." The gels consist of discontinuous gradients of varying acrylamide concentrations, as indicated by the solid horizontal lines. The dashed lines correspond to the migration distance of two marker proteins. Approximately 225 μg of protein were applied to each gel. Marker proteins were obtained from commercial sources, and about 20 μg of each were applied to a gel.
"Methods." The electrophoretic patterns of the TX-soluble and TX-insoluble fractions, each comprising about 50% of the total SER protein, are shown in Fig. 1. These patterns are similar to those published previously (3), which showed that the two TX subfractions have some protein bands in common and others which are limited to one fraction only. The percentage of NADPH cytochrome c reductase and cytochrome P-450 activity released into the TX-soluble fraction was determined for SER from untreated rats. SER which had been suspended by sonication in 1% TX was examined for enzyme activity before and after centrifugation to remove the TX-insoluble material. All of the cytochrome c reductase and about 43% of the cytochrome P-420 activity present in the sonicated SER were recovered in the TX-soluble fraction.

A major protein band, designated as Band C (Fig. 1), is apparent in both TX subfractions. With proteins of known molecular weight markers, the molecular weight of this band was estimated to be about 50,000. Several lines of evidence suggest that this band contains the CO-binding protein, cytochrome P-450: based on an estimated molecular weight of 50,000, this protein comprises 4 to 5% of the microsomal protein and up to 20% of phenobarbital-stimulated microsomes (1, 24). As noted above, about 43% of the cytochrome P-450 activity measured in SER is released into the TX-soluble fraction, which agrees well with the distribution of radioactivity found in Band C of the two TX subfractions (Fig. 3).

To confirm further that Band C contains cytochrome P-450, we partially purified the solubilized form of this protein, cytochrome P-420. As indicated under "Methods," this fraction contains a heme protein and migrates as a single band on nondenaturing polyacrylamide gel at pH 10.0. This fraction showed CO-binding activity at 420 nm, although its specific CO-binding activity was only slightly greater than the specific CO-binding activity of fresh SER. The failure to obtain a significant enrichment of the specific CO-binding activity of the cytochrome P-420 fraction is thought to result from progressive loss of CO-binding activity of the protein once it is removed from the membrane, as has been reported earlier (17). The SDS gel electrophoretic pattern of the partially purified cytochrome P-420 fraction is shown in Fig. 2. The bulk of the protein in this fraction migrates in the 50,000 molecular weight range, although contaminating protein bands are evident. The effect of snake venom digestion on the molecular weight distribution of TX-insoluble SER, also shown in Fig. 2, appears to be negligible, indicating that little if any proteolysis occurs during the 24-hour incubation with heat-treated snake venom. Thus we can rule out the possibility that the 50,000 molecular weight protein in the cytochrome P-420 fraction was derived from a larger polypeptide in the TX-insoluble SER fraction.

Amino Acid Incorporation into SER Proteins following Drug Administration—The effect of drug administration on the synthesis of microsomal proteins was studied by comparing incorporation of [3H]leucine into drug-induced SER proteins with incorporation of [3H]leucine into uninduced SER proteins fractionated on SDS polyacrylamide gels. The variability inherent in an experiment of this type was determined by a control experiment in which neither the 3H- nor 14C-labeled animal received drug treatment. The data from this experiment is shown in Fig. 3. The major peak of radioactivity in both TX subfractions (middle frame) corresponds to Band C in Fig. 1.

The effect of phenobarbital on amino acid incorporation into SER proteins is shown in Fig. 4. Amino acid incorporation into Band C is stimulated relative to other protein bands in both TX subfractions, indicating that most of the compositional change in microsomal proteins in response to phenobarbital is associated with protein (or proteins) in Band C. In this and subsequent figures, the correspondence between Band C on the optical gels and the Band C on the [3H]:[14C] ratios plot is assured by the method of matching the two plots (see "Methods"). Thus the [3H]:[14C] ratios reported for Band C correspond to the [3H]:[14C] ratios in the region of peak radioactivity. This may include cytochrome P-450 as well as other phenobarbital-induced proteins. Since all of the NADPH cytochrome c reductase is released into the TX-soluble fraction, this enzyme does not contribute to Band C in the TX-insoluble fraction. In order to verify that cytochrome P-450 contributes, either totally or partially, to the observed increase in amino acid incorporation into Band C, the physiological response of isolated cytochrome P-420 to phenobarbital administration was assessed by a double label experiment.
Fig. 3. Electrophoretic patterns of radioactivity and optical density of the TX-soluble (left) and TX-insoluble (right) SER subfractions. One animal was given 250 μCi of [3H]leucine and another animal, 75 μCi of [14C]leucine 4 hours before killing. The SER fractions from the two animals were combined and electrophoretically treated as described under "Methods." Approximately 225 μg of protein were applied to the small analytical gels and 5 mg of protein to the preparative size gels. The upper box shows the optical gel scan of the stained analytical gels; the two similar to the one described above. Using the drug and isotope doses and schedule described in Fig. 4, the relative rates of amino acid incorporation into the cytochrome P-420 fraction and total SER were determined. The [%H:14C] ratio for the P-420 fraction was 2.4. This [%H:14C] value was similar to those found for average SER proteins and Band C, respectively, seen in Fig. 4. In view of the significant percentage of total SER protein which cytochrome P-420 comprises, a marked increase in the rate of synthesis of this protein would be expected to result in a marked increase in amino acid incorporation into Band C relative to other phenobarbital-induced proteins. Results of the isolation of NADPH cytochrome c reductase described by Omura et al. (25) indicated that this enzyme constitutes about 0.6% of washed microsome protein, whereas the amount of cytochrome P-450 is several times greater. Estabrook and Cohen (24) have estimated the stoichiometry of the mixed function oxidase system to be several moles of cytochrome P-450 to 1 mole of reductase. A comparison of [%H:14C] ratios in the two TX subfractions shows that the increase in [%H:14C] ratios in the 50,000 molecular weight band is greater in the TX-insoluble fraction. In all of the double label experiments performed, we found that the preferential increase (or decrease) in [%H:14C] ratios in Band C was more pronounced in the TX-insoluble fraction (Figs. 4 to 7). This finding is presumably related to the difference in the relative amounts of cytochrome P-450 in Band C between the two TX subfractions.

Fig. 4 also shows that proteins in the molecular weight range greater than 50,000 generally have higher [%H:14C] ratios than protein bands in the molecular weight range less than 50,000. Since at least one other microsomal protein, NADPH cytochrome c reductase, and the activities of a wide variety of drug metabolism reactions are induced by phenobarbital, the general elevation in [%H:14C] ratios among larger protein subunits may reflect phenobarbital induction of these proteins. However, the true [%H:14C] base-line value, i.e. the ratio of a protein whose synthesis is unaffected by drug administration, cannot be determined from these results. The SER fractions from the two animals were combined on the basis of equivalent volumes of liver homogenate rather than equal protein basis. Thus, the absolute [%H:14C] ratios are dependent on the relative amounts of SER per volume of liver homogenate, the relative specific activities of the amino acid pools in the livers, and the relative rates of protein synthesis. The experiments described here measure only the relative rates of protein synthesis.

The results of a typical experiment in which 3-methylcholanthrene was administered to the [%H-labeled animal are shown in Fig. 5. The [%H:14C] patterns of the SER subfractions differ in several respects from the data in Fig. 4. Amino acid incorporation into the protein band identified with cytochrome P-450 is only slightly increased, consistent with the increase in cytochrome P-450 activity shown in Table I. The [%H:14C] ratios of the large molecular weight proteins are similar to proteins of smaller molecular weight. In the lower molecular weight region a few
FIG. 4. Relative amino acid incorporation into TX-soluble (left) and TX-insoluble (right) SER proteins in response to phenobarbital administration. The experimental animal was injected intraperitoneally with 100 mg per kg of phenobarbital dissolved in water, and a control animal received a corresponding volume of water 16 hours prior to killing. Four hours before killing the experimental animal received 250 μCi of [3H]leucine and the control received 75 μCi of [14C]leucine. The SER fractions from each animal were combined and electrophoretically treated as described under "Methods." Approximately 225 μg of protein were applied to the analytical gels and 5 mg of protein to the preparative size gels. The 3H:14C ratios were matched to the optical scan as described in the text. The results are typical of two such experiments.

bands in the TX-insoluble fractions show increased amino acid incorporation.

The effect of hematin on the short term labeling pattern of SER proteins is shown in Fig. 5. This data illustrates some interesting contrasts to the data on phenobarbital induction (Fig. 4). Whereas phenobarbital results in a uniform increase in the microsomal enzymes assayed, hematin causes the same enzymes to decrease uniformly (Table I). Accordingly, the protein band identified with cytochrome P-450 shows a preferential decrease in amino acid incorporation, and those proteins with molecular weights greater than 50,000 have in general lower 3H:14C ratios than the smaller molecular weight protein subunits. These findings suggest that the group of enzymes, particularly cytochrome P-450, whose incorporation into microsomes is stimulated by phenobarbital, is coordinately decreased by hematin.

The results of the experiment in which the 3H-labeled animal was given phenobarbital and the 14C-labeled animal was given hematin and phenobarbital are shown in Fig. 7. If hematin is effective in preventing microsomal enzyme induction by phenobarbital, as indicated in Table I, the 3H:14C ratios obtained in

Fig. 6. Relative amino acid incorporation into TX-soluble (left) and TX-insoluble (right) SER proteins following hematin administration. Hematin, dissolved in 10 mM KOH, was administered to an experimental animal at a dose (16 mg per kg) 18, 16, and 4 hours prior to killing; the control animal received a corresponding volume of 10 mM KOH at the same time intervals. Four hours prior to killing, the hematin-treated animal was injected with the 250 μCi of [3H]leucine and the control, with 75 μCi of [14C]leucine. The procedures used are identical with those described in Fig. 4.
this experiment should resemble those in which phenobarbital stimulation was compared to no drug treatment (Fig. 4). This, in fact, was found. The \(^{3}H:^{14}C\) ratio of Band C, which is somewhat higher than the corresponding ratio in the experiment shown in Fig. 4, may be due to the additive effect of increased \(^{3}H\) incorporation in response to phenobarbital (Fig. 4) and decreased \(^{14}C\) incorporation due to hematin inhibition, as indicated in Fig. 6.

**DISCUSSION**

The experiments described in this paper were designed to investigate the relative rates of synthesis of microsomal proteins in response to various pharmacological stimuli. Particular attention was focused on protein Band C, a 50,000 molecular weight protein band as displayed on SDS acrylamide gels. We have shown that this band contains cytochrome P-450 and that changes in the measurable activity of cytochrome P-450 in response to the three compounds administered are closely paralleled by changes in the amino acid incorporation into Band C. Although the data do not indicate the extent to which proteins other than cytochrome P-450 contribute to Band C, the double label experiment showing an increase in the amino acid incorporation into cytochrome P-420 following phenobarbital administration showed that cytochrome P-450 contributes, at least in part, to the increase in amino acid composition in Band C in response to phenobarbital.

Phenobarbital, 3-methylcholanthrene, and hematin also have characteristic effects on the rate of synthesis of SER proteins other than those contained in Band C. Phenobarbital stimulates the synthesis of several large molecular weight (greater than 50,000) proteins in both of the TX subfractions of SER, whereas the observed effect of 3-methylcholanthrene is confined to a few smaller molecular weight (less than 50,000) proteins in the TX-insoluble SER fraction. Numerous studies have emphasized differences between the induction of SER by phenobarbital and 3-methylcholanthrene with respect to the mechanism of induction (26, 27), enzymes induced (5, 6), and changes in cell morphology (7, 28). The present study indicates that the set of microsomal proteins induced by phenobarbital overlaps very little with the set of proteins induced by 3-methylcholanthrene. In the case of induction by phenobarbital, it can be argued that some of the larger molecular weight proteins which appear to be induced in fact are aggregates of cytochrome P-450 or undissociated membrane particles containing cytochrome P-450. We cannot rule out this possibility if cytochrome P-450 is covalently attached to other membrane proteins; however, we have shown by a variety of methods that higher molecular weight bands seen on the gels are not protein aggregates (3).

The data on microsome induction by phenobarbital and 3-methylcholanthrene showing an increase in the \(^{3}H:^{14}C\) ratios of particular SER proteins indicate that these drugs act by increasing the rate of synthesis of some membrane proteins. Although a decrease in the rate of protein degradation would result in an accumulation of newly synthesized protein, it is unlikely that the corresponding \(^{3}H:^{14}C\) ratio would increase significantly 4 hours after isotope labeling. Therefore, if decreased protein degradation contributes to microsomal induction, the experiments described here would probably not detect it.

That the rate of synthesis of some microsomal proteins is increased in response to phenobarbital and 3-methylcholanthrene has been shown in a variety of studies. Several workers, studying the effects of drugs on protein synthesis in vitro (1, 2) and in vitro (29-31) have shown that the rate of amino acid incorporation into microsomal proteins increases in response to phenobarbital and 3-methylcholanthrene. Several laboratories have shown that phenobarbital stimulates the synthesis of NADPH cytochrome c reductase (1, 2, 11). In the present study we show that another component of the mixed function oxidase system, cytochrome P-450, also has a significantly increased rate of synthesis in response to phenobarbital.

The decrease in \(^{3}H:^{14}C\) ratios of some SER proteins following hematin administration can likewise be ascribed to decreased synthesis rather than increased degradation. Marver has proposed that hematin acts to prevent the synthesis of heme proteins through the inhibition of heme synthesis, specifically at the level of \(\delta\)-aminolevulinic acid synthetase (19, 20). Support for this proposal comes from the study of Raisfeld et al. (32) showing that the inhibition of heme synthesis by 5-aminotriazole prevented induction of cytochrome P-450 by phenobarbital. However, this hypothesis does not explain why the exogenously supplied heme cannot be utilized in heme protein synthesis. An alternative proposal is that hematin inhibits the incorporation or assembly of newly synthesized cytochrome P-450 into endoplasmic reticulum, possibly as a result of enhanced lipid peroxidation. Studies by Tappel (33), Hatefi (34), and others (35-37) have pointed out the liability of microsomes to lipid peroxidation damage in vitro. Among the most efficient catalysts of lipid peroxidation are iron and iron-coordinated compounds such as hematin. Cytochrome P-450 would appear to be particularly vulnerable to lipid peroxidation damage, since it is associated with phospholipid (12), as well as the oxygen binding site of the mixed function oxidase system.

The question of how membrane proteins are incorporated into, and removed from, previously existing microsomes in response to drug-induced synthesis and degradation is related to the question of how membrane proteins are replaced in the steady state. A number of studies have shown that the protein components of endoplasmic reticulum have heterogeneous turnover rates (1-3), thus implying that proteins are continually being added to and removed from previously existing membranes. We have previously proposed a model in which membranes are assembled from and degraded as cytoplasmic components. This proposal was based in part on our finding that the protein components of SER and plasma membranes show the same correlation between subunit size and degradation rates as do the supernatant proteins (3). An alternative explanation for the genesis of membranes would have membrane proteins synthesized by membrane-bound ribosomes, such that an intermediate (cytoplasmic) pool of membrane proteins need not exist. Although such an explanation is supported by studies showing that pulse-labeled cytochrome c reductase of the endoplasmic reticulum first appears in rough endoplasmic reticulum, and then only later appears in the smooth endoplasmic reticulum (38), Omura and Kuriyama (39) have recently found that such a process does not occur with the bulk of membrane proteins.

We are currently involved in a study to differentiate between these two models. Using an antibody to the partially purified cytochrome P-420, immunological studies aimed at determining whether a cytoplasmic pool of cytochrome P-450 exists are in progress. If such a pool exists, the nature of this pool during increased and decreased rates of membrane synthesis (assembly)
as effected by pharmacological agents should elucidate further the mechanism of membrane biosynthesis and degradation.

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