Identification of the Cytochrome P-450 Induced by Macrolide Antibiotics in Rat Liver as the Glucocorticoid Responsive Cytochrome P-450$_p$[†]

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Received August 6, 1984

ABSTRACT: We administered triacetyloleandomycin (TAO) to rats and found that this macrolide antibiotic is the most efficacious inducer of liver microsomal cytochrome P-450 (P-450) examined to date. Liver microsomes prepared from TAO-treated rats contained greater than 5.0 nmol of P-450/mg of protein and a single induced protein as judged by analysis on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. This protein comigrated with P-450$_p$, the major form of P-450 induced in liver microsomes of rats treated with pregnenolone-16α-carbonitrile (PCN) or dexamethasone (DEX). On immunoblots of such gels developed with antibodies to P-450$_p$, the TAO-induced protein reacted strongly as a single band. There was strict parallelism between (a) the amount of immunoreactive P-450$_p$ in liver microsomes prepared from untreated rats or from rats treated with phenobarbital, TAO, DEX, or PCN, (b) the ability of these microsomes to catalyze conversion of TAO to a metabolite which forms a spectral complex, and (c) the ethylmorphine and erythromycin demethylase activities. Antibodies to P-450$_p$ specifically blocked microsomal TAO metabolite complex formation and ethylmorphine and erythromycin demethylase activities. Moreover, anti-P-450$_p$ antibodies completely immunoprecipitated solubilized TAO metabolite complexes prepared by detergent treatment of liver microsomes obtained from TAO-treated rats. Finally, we found that the major form of P-450 isolated from liver microsomes of TAO-treated rats and purified to homogeneity was indistinguishable from purified P-450$_p$ as judged by molecular weights, spectral characteristics, enzymatic activities, ability to bind TAO, peptide maps, and amino-terminal amino acid sequences. We concluded that, in addition to glucocorticoids, macrolide antibiotics are specific inducers of P-450$_p$.

The liver responds to the presence of drugs or other foreign compounds by increasing or decreasing (or both) the concentrations of the cytochromes P-450, a multigene family of microsomal hemoproteins that catalyze the biooxidation of numerous foreign and endogenous substrates (Snyder & Remmer, 1979; Guengerich et al., 1982). It has been customary to classify compounds as resembling either phenobarbital or 3-methylcholanthrene, two prototype inducers that increase the liver concentrations of different forms of the hepatic cytochromes P-450. Recently, we proposed that glucocorticoids and anti-glucocorticoids like pregnenolone-16α-carbonitrile (PCN)[1] constitute a "third class" of inducers that regulate yet another unique form of cytochrome P-450, P-450$_p$[2] (Heuman et al., 1982; Schuetz et al., 1984; Schuetz & Guzelian, 1984a). Even this simple classification is inadequate because there are well-characterized forms of cytochrome P-450 for which no inducer has been identified (Cleveland et al., 1977; Cheng & Schenkman, 1982; Kamataki et al., 1983; Ryan et al., 1984) and other forms that appear to be induced by agents not represented in one of these three groups (Tamburini et al., 1984; Past & Cook, 1982; Koop et al., 1982).

Among the most efficacious inducers of cytochrome P-450 are the macrolide antibiotics exemplified by TAO (Bonfils et al., 1982; Delaforge et al., 1983). These compounds may alter drug metabolism in human liver and have been implicated in a number of clinically important adverse drug interactions (Weinberger et al., 1977; Szefler et al., 1982; Larrey et al., 1983). Investigations of this phenomenon have, to date, largely been restricted to spectral studies. These have revealed that TAO is an inducer of total carbon monoxide binding hemoprotein in liver microsomes from rabbits (Bonfils et al., 1982), rats (Delaforge et al., 1983), and humans (Pessayre et al., 1982). TAO also serves as a substrate for an N-oxidation reaction catalyzed by the same form of cytochrome P-450 that is induced by TAO (Bonfils et al., 1982; Pessayre et al., 1982). The TAO metabolite binds strongly to the reduced iron of the heme prosthetic group of this cytochrome resulting in the formation of a persistent metabolic intermediate complex (Pessayre et al., 1981). From this information it has been proposed that TAO may act not only as an inducer but also through its metabolite, as a competitive inhibitor of the cytochrome. To test this hypothesis and to better understand the interactions of TAO with the cytochrome P-450 system,

[†] This research was supported in part by Grants P02-AM18976-08 and ES01519-08 from the National Institutes of Health, Research and Career Development Award K04-AM-00570 from the National Institutes of Health (P.S.G.), Training Grant 5T32-AM-07150-9 from the National Institutes of Health (P.B.W.), Fogarty Institute Fellowship Award 3F05TW03386-01S1 (P.M.), Virginia Environmental Endowment Award 83-12, and Award J-53 from the Jeffress-Gwathmey Trust (S.A.W.).

[1] Abbreviations: P-450$_b$ and P-450$_c$, the major forms of rat liver cytochrome P-450 purified from animals treated with phenobarbital or 3-methylcholanthrene, respectively (Ryan et al., 1984); PCN, pregnenolone-16α-carbonitrile; DEX, dexamethasone; TAO, triacetyloleandomycin; TAO complex, a spectrally detectable, metabolic intermediate species consisting of a TAO metabolite bound to the reduced (ferrous) iron of cytochrome P-450 heme; EDTA, ethylenediaminetetraacetic acid; BNF, β-naphthoflavone; SDS, sodium dodecyl sulfate; Me_2SO, dimethyl sulfoxide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] In previous reports we have used the designations "P-450$_{PCN}$" and "P-450$_{DEX}$" to denote the major forms of hepatic cytochrome P-450 purified from rats pretreated with pregnenolone-16α-carbonitrile or dexamethasone, respectively. Since the evidence to date indicates that these cytochromes are the same (Schuetz et al., 1984), we have used in this report the term "P-450$_p$" to refer to this steroid-inducible hemoprotein. "P-450$_{TAO}$" refers to the major form of hepatic cytochrome P-450 purified from rats treated with triacetyloleandomycin.

0006-2960/85/0424-2171$01.50/0

it would be desirable to define biochemically the form (or forms) of the hemoprotein induced by TAO. Recent studies in the rabbit have suggested that TAO induces one of the LM3 forms of cytochrome P-450 originally purified from untreated animals (Bonfils et al., 1983). In the present study, we have isolated, purified, and characterized the major form of hepatic cytochrome P-450 from rats treated with TAO. With the use of a battery of biochemical and immunochemical tests we demonstrate that P-450$_{TAO}$ is indistinguishable from the glucocorticoid inducible cytochrome, P-450$_p$.

Materials and Methods

Materials. PCN, TAO, and erythromycin were gifts from Upjohn Co. (Kalamazoo, MI), Pfizer, Inc. (New York, NY), and Laboratoires Roussel (Paris), respectively. Dex, ethylmorphine, dilauroylphosphatidylcholine, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO); α-chymotrypsin, *Staphylococcus aureus* V8 protease, rabbit anti-goat IgG, and goat peroxidase anti-peroxidase were from Miles (Elkhart, IN); nitrocellulose sheets were from D and L Filter Corp. (Woburn, MA); 3,3′-diaminobenzidine tetrahydrochloride was from Pfaltz & Bauer (Stamford, CT).

Animals and Treatments. Male and female Sprague-Dawley rats weighing 150–200 g were obtained from Flow Labs (Dublin, VA) and maintained in pairs in wire bottom cages with free access to chow and water. PCN and DEX suspended in water containing 2% Tween 80 were given by lavage (300 mg kg^{-1} day^{-1} for 4 days). TAO was thoroughly mixed into the ground chow (1.5 g/100 g of chow). Rats were fed this diet for 10 days. Phenobarbital was given in 0.1% solution in drinking water for 6 days; BNF was given intraperitoneally at a dose of 50 mg kg^{-1} day^{-1} in corn oil for 5 days.

Purification of P-450$_{TAO}$ and P-450$_p$. After an overnight fast, the animals were killed by decapitation, and microsomes were prepared and stored as previously reported (Elshourbagy & Guzelian, 1980). P-450$_p$ was purified from PCN/DEX-treated female rats by a previously described procedure (Elshourbagy & Guzelian, 1980), and P-450$_{TAO}$ was purified from TAO-treated male rats by the following procedure. Liver microsomes were diluted to 2.5 mg of protein/mL of 0.1 M potassium phosphate buffer (pH 7.6), supplemented with 1 mM EDTA, 20 μM butylhydroxytoluene, and 20% glycerol. Sodium cholate (20% solution) was added dropwise with stirring at 4 °C to achieve final cholate concentration of 0.7%. The solution of solubilized microsomes was stirred an additional 30 min and centrifuged at 105000g for 1 h, and the supernatant was applied to an aminooctylamino-Sepharose 4B column (250 mL), which had been equilibrated in a 0.1 M potassium phosphate buffer (pH 7.25), containing 1 mM EDTA, 0.42% cholate, and 20% glycerol. Cytochrome P-450 was eluted with 2 L of 0.1 M potassium phosphate buffer, pH 7.25, supplemented with 1 mM EDTA, 0.33% cholate, 0.06% Lubrol PX, and 20% glycerol. For each fraction (4 mL) absorbance was recorded at 280, 417, and 457 nm to monitor protein, hemoprotein, and TAO complex, respectively. The profiles of absorbance at all three wavelengths were identical with a unique sharp peak eluting after 560 mL. The 12-peak fractions displaying greater than 0.5 OD at 457 nm and ratio of absorbance 457/417 in the range 1.0–1.4 were combined (50 mL), dialyzed overnight against 2 L of 10 mM potassium phosphate buffer containing 0.1 mM EDTA and 20% glycerol, and applied to a hydroxylapatite column (15 mL) previously equilibrated with the dialysis buffer containing 0.2% Lubrol PX. The column was washed with 200 mL of equilibration buffer and 200 mL of 45 mM potassium phosphate equilibration buffer followed by 200 mL of 90 mM potassium phosphate equilibration buffer. P-450$_{TAO}$ was then eluted with 180 mM potassium phosphate equilibration buffer. In this P-450$_{TAO}$ fraction, 45% of total CO binding hemoprotein was in the form of the TAO complex. This fraction was dialyzed overnight against the hydroxylapatite column equilibration buffer and applied to a second hydroxylapatite column (10 mL). Detergent was removed by extensive washing of the column with 500 mL of 10 mM potassium phosphate buffer containing 0.1 mM EDTA and 20% glycerol. The cytochrome was then eluted as a sharp peak with 400 mM potassium phosphate buffer and dialyzed overnight against 50 mM potassium phosphate buffer, pH 7.4, containing 20 μM EDTA and 20% glycerol. All the purification steps were carried at 4 °C.

Preparation of Anti-Cytochrome P-450 Antibodies. Antibodies to purified P-450$_p$, P-450$_b$, and P-450$_c$ were raised in goats. IgG was isolated by fractionation of serum with ammonium sulfate followed by chromatography on DEAE-cellulose (Elshourbagy & Guzelian, 1980). "Form-specific" anti-P-450 IgG and nonimmune IgG were prepared by absorption against immobilized liver microsomes prepared from appropriately treated rats as described elsewhere (Elshourbagy et al., 1981).

TAO Complex: Detection, Quantitation, and Formation in Vitro. Liver microsomes from TAO-treated rats were diluted to 1 mg/mL in 0.1 M potassium phosphate buffer, pH 7.4, and the solution (10 mL) was divided in three aliquots, a–c. An equal volume (750 μL) of aliquot a was placed in both sample and reference cuvettes of an Aminco DW2 spectrophotometer, and the base line was recorded; 10 μL of a 2 mM K_3FeCN_6 solution was then added to the reference cuvette and 10 μL of water to the sample cuvette. After 10 min a difference spectrum displaying a peak of 457 nm and a trough at 415 nm was recorded, indicating that the P-450$_{TAO}$ metabolite complex, present in the liver microsomes of TAO-treated rats, had been destroyed in the reference cuvette by the addition of ferricyanide. The absorbance difference between 457 and 510 nm ($\Delta A^{457/510}$) was recorded. Ferricyanide (25 μM final concentration) was added to aliquot b and an equal volume of water to aliquot c. After 10 min cytochrome P-450 concentration was determined in both aliquots b and c as the reduced CO binding hemoprotein according to the method of Omura & Sato (1964). The extinction coefficient for absorbance of the TAO complex was determined as follows: $\Delta\Sigma^{(457/510)} = \Delta A^{(457/510)}/(b - c)$ where $\Delta A^{(457/510)}$ represented the absorbance recording from aliquot a, b, the total cytochrome P-450 concentration in aliquot b, and c, the uncomplexed cytochrome P-450 concentration as measured in aliquot c. In agreement with others (Delaforge et al., 1983), we found $\Delta\Sigma^{(457/510)} = 68$ mM^{-1} cm^{-1}.

In some experiments ferricyanide-treated TAO microsomes (referred to as uncomplexed microsomes) were tested for the ability to re-form in vitro the TAO complex. Microsomes were diluted to 1 mg/mL with 0.1 M potassium phosphate buffer and treated with ferricyanide for 10 min at room temperature. Equal amounts of the solution (750 μL) were placed in both sample and reference cuvettes heated to 35 °C in an Aminco DW2 spectrophotometer. A base line was recorded, and 3 μL of 5 mM TAO in Me_2SO was added to the sample cuvette and equal volume of the solvent alone to the reference cuvette. (This concentration of TAO yields the maximal TAO complex.) Five minutes later a type I binding spectrum was recorded (peak at 390 nm and trough at 420 nm). NADPH (final concentration 1 mM) was then added to both cuvettes, and repetitive scanning between 360 and 510 nm was imme-

diately started. After a 2-min lag a peak of absorbance at 457 nm appeared and increased linearly (with a concomitant decrease at 415 nm) for 10–15 min. A stable plateau of absorbance was observed after approximately 30 min. Under these conditions, the maximal level of the TAO complex formed in vitro was within 10% of the level of complex formed in vivo determined directly with freshly isolated TAO microsomes.

Demethylation of Ethylmorphine and Erythromycin. Purified P-450$_{TAO}$ or P-450$_p$ (0.5–1 nmol), 30 μg of sonicated dilauroylphosphatidylcholine, and 2–3 nmol of NADPH-cytochrome P-450 reductase were incubated for 5 min at 25 °C in a small volume (<100 μL) of 0.05 M potassium phosphate buffer, pH 7.4. Then the substrate (erythromycin, 0.4 μmol, or ethylmorphine, 1 μmol) in 0.1 M Tris-HCl buffer was added to a final volume of 1 mL. The reactions were initiated by addition of NADPH (0.5 mM final concentration) and were incubated at 37 °C for 10 min. Formaldehyde formation was detected by the method of Nash (Nash, 1953). Microsomes (1 mg of protein/mL) were assayed in a similar manner except the buffer was 0.1 M potassium phosphate, pH 7.4.

Polyacrylamide Slab Gel Electrophoresis and Immunoblot Analysis. Analysis of microsomes or purified proteins by polyacrylamide (10%) slab gel electrophoresis was carried out at room temperature in the presence of SDS, according to standard methods (Laemmli, 1970). The resolved proteins either were stained with silver (Merril et al., 1979) or were electrophoretically transferred to a nitrocellulose sheet for immunoblot analysis (Guengerich et al., 1982). The nitrocellulose sheet was sequentially incubated with 10% calf serum plus 3% bovine serum albumin, 0.1 mg/mL anti-P-450$_p$ IgG, rabbit anti-goat IgG, goat peroxidase anti-peroxidase, and, finally, 50 mM Tris-HCl buffer, pH 7.5, containing hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride (Guengerich et al., 1982).

Inhibition of Monooxygenase Activities by Anti-Cytochrome P-450 Antibodies. Microsomes were diluted to a final concentration of carbon monoxide binding hemoprotein of 1 μM and were incubated with antibody (0–10 mg of IgG/mL) for 15 min at 4 °C and 15 min at 37 °C. Then in vitro formation of the TAO complex and erythromycin and ethylmorphine demethylase activities were measured as described in preceding sections.

Immunoprecipitation of the TAO Complex Formed in Vivo. Liver microsomes prepared from a TAO-treated male rat (5.2 nmol of cytochrome P-450/mg of protein) were diluted to 5 mg of protein/mL in a 0.1 M potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.1 mM dithiothreitol, and 20% glycerol in a final volume of 5 mL. The microsomes were solubilized by the addition of 25 μL of 20% sodium cholate followed by 10 μL of Nonidet NP-40, and the mixture was stirred for 30 min at 4 °C. The solution was then centrifuged at 105000*g* for 30 min and the supernatant collected. Under these conditions 100% of the spectrally measured TAO complex initially present in microsomes was recovered in the solubilized material. Aliquots of solubilized microsomes (0.5–1 μM TAO complex) were then incubated for 30 min at 25 °C in 50 mM potassium phosphate buffer, pH 7.4, containing 0.2% NP-40, 1 mM EDTA, and 0.1 M KCl, in the presence of an increasing amount of antibody (from 0 to 25 mg of IgG protein/mL). *S. aureus* ghosts (10 μL of a 10% solution) were added, and after 15 min the immunoprecipitate was sedimented by centrifugation at 10000*g* for 2 min. The TAO complex remaining in the supernatant was quantified by difference spectroscopy (see preceding section).

Peptide Maps. Peptide mapping was performed according to published methods (Cleveland et al., 1977). P-450$_{TAO}$ or P-450$_p$ was diluted to 0.25 mg/mL in a 0.125 M Tris-HCl buffer (pH 6.8) containing 0.5% SDS and 10% glycerol, and the solution was heated to 100 °C for 2 min. *S. aureus* V8 protease or α-chymotrypsin was then added (6.25 μg/mL) and the mixture was incubated 30 min at 37 °C. The reaction was quenched with 10% β-mercaptoethanol and 2% SDS. This mixture was heated at 100 °C for 2 min, and 10 μg of each proteolytic digest was subjected to electrophoresis in 15% polyacrylamide slab gels. The resolved peptides were transferred electrophoretically to a nitrocellulose sheet and were visualized either by silver staining or by immunoblot analysis using anti-P-450$_p$ IgG.

NH_2-Terminal Sequence Analysis. Edman degradations were performed with a Beckman 890C sequencer equipped with a cold trap. For automatic sequencing, approximately 20 nmol of protein was applied with the standard sample application subroutine. Edman degradation was then performed with the 0.1 M Quadrol program (Beckman program no. 121078). The released thiazolinone derivatives were converted to phenylthiohydantoins (PTH) by treatment with 1 N HCl in methanol at 50 °C for 10 min. The PTH derivatives were then identified by high-performance liquid chromatography on a Beckman column. The PTH derivatives were eluted with a linear gradient between solvent A (5% tetrahydrofuran and 95% 5.25 mM sodium acetate, pH 5.02) and solvent B (10% tetrahydrofuran and 90% acetonitrile). A gradient from 0 to 40% solvent B in 20 min at 1.3 mL/min was used. The elution was monitored at 269 nm. Alternatively, the PTH derivatives were hydrolyzed with HI and identified by amino acid analysis (Peterson, 1981).

Results

Characterization of the Cytochrome P-450 Induced by TAO in Liver Microsomes. We prepared liver microsomes from rats fed a standard diet containing TAO and found, in agreement with others (Delaforge et al., 1983), a spectacular increase in total cytochrome P-450 concentration. The values in the microsomes obtained from TAO-treated rats were higher (greater than 5.0 nmol/mg of protein) than those previously reported (Guengerich et al., 1982) for any other inducer and represented more than a 5-fold increase over the basal values in untreated rats (0.7–1.0 nmol/mg of protein). However, this dramatic induction of cytochrome P-450 by TAO would go unrecognized if CO binding hemoprotein were measured by the standard spectrophotometric assay in dithionite-reduced microsomes. This is because 70% of the cytochrome P-450 in microsomes prepared from TAO-treated rats is present as a stable complex which consists of an oxidized metabolite of TAO (presumably a nitroso derivative) bound tightly to the reduced iron of the heme prosthetic moiety of the cytochrome (Pessayre et al., 1981). This TAO metabolite–cytochrome P-450 complex (TAO complex) is catalytically inactive and is incapable of binding CO even if the microsomes are first reduced by addition of dithionite. However, if the heme iron is first oxidized by exposure of the microsomes to potassium ferricyanide, the TAO complex is destroyed, monooxygenase activities of microsomes are restored, and the full content of CO binding hemoprotein is revealed (data not shown).

We compared the pattern of microsomal proteins from TAO-treated rats separated by SDS–polyacrylamide gel electrophoresis to those of microsomes isolated from rats treated with phenobarbital, BNF, PCN, or DEX. Microsomes from TAO-treated rats contained a prominent band coincident in migration with a major band of aproximately 51 000-daltons

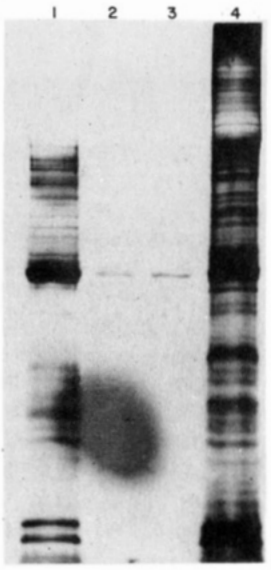

FIGURE 1: Electrophoretic profile of rat liver microsomes and purified cytochromes P-450. Liver microsomes and purified cytochromes were prepared as described under Materials and Methods and were subjected to electrophoresis on a SDS–10% polyacrylamide slab gel and visualized by silver staining. (Lanes 1 and 4) 8 μg of liver microsomal protein prepared from TAO- and PCN-treated animals, respectively. (Lanes 2 and 3) 0.5 μg of purrified P-450$_{TAO}$ and P-450$_p$, respectively.

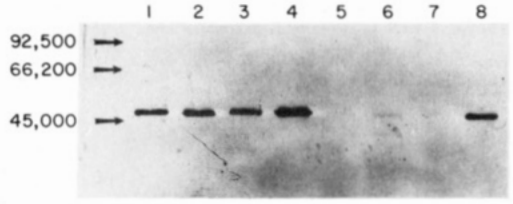

FIGURE 2: Immunoblot of liver microsomes and purified cytochromes P-450 developed with anti-P-450$_p$ IgG. Protein samples were resolved by electrophoresis in a SDS–10% polyacrylamide gel and transferred electrophoretically to nitrocellulose. The blot was developed with anti-P-450$_p$ IgG and visualized by peroxidase staining (see Materials and Methods). (Lane 1) 0.75 μg of P-450$_p$; (lane 2) 5 μg of PCN/DEX microsomes; (lane 3) 0.75 μg of P-450$_{TAO}$; (lane 4) 3 μg of TAO microsomes. The remaining lanes contained 10 μg each of microsomes prepared from an untreated female (lane 5) and untreated male (lane 6), a BNF-treated male (lane 7), and a phenobarbital-treated male (lane 8).

present in microsomes isolated after PCN (Figure 1) or DEX treatment (not shown). We have shown previously that this band represents a unique form of cytochrome P-450 (P-450$_p$) which is induced by treatment of rats with PCN, DEX, or PCN plus DEX (PCN/DEX) (Schuetz et al., 1984; Schuetz & Guzelian, 1984a,b; Elshourbagy & Guzelian, 1980; Heuman et al., 1982). When the electrophoretically resolved microsomal proteins were transferred to nitrocellulose and incubated with anti-P-450$_p$ antibodies, microsomes from rats treated with TAO or PCN/DEX exhibited a single immunoreactive protein with mobility identical with that of purified P-450$_p$ (Figure 2). This protein was absent from microsomes from untreated female rats and BNF-treated male rats but was induced in microsomes isolated from phenobarbital-treated male rats (Figure 2). These results confirm that phenobarbital is an inducer of P-450$_p$ (Guengerich et al., 1982; Heuman et al., 1982). Although there may appear to be slight molecular weight differences between the various immunoreactive proteins in Figure 2, repetitive immunoblot analyses in which the preparations were compared in adjacent wells and SDS–polyacrylamide gels such as Figure 1 demonstrate that the immunoreactive proteins have the same molecular weight. In

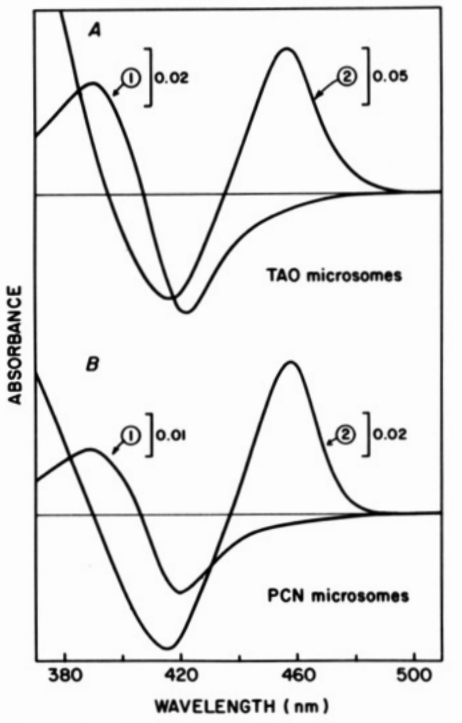

FIGURE 3: Formation of TAO complex in vitro by liver microsomes from TAO- or PCN-treated rats. (A) A suspension of liver microsomes prepared from TAO-treated female rat (1 mg of protein/mL in 0.1 M potassium phosphate buffer, pH 7.6) was preincubated with potassium ferricyanide to dissociate the TAO complex formed in vivo and then divided into two cuvettes of an Aminco DW2 spectrophotometer at 35 °C. After a base line was recorded, then 3 μL of a 5 mM TAO solution in Me_2SO and 3 μL of pure Me_2SO were added to the sample and reference cuvettes, respectively. Spectrum 1 was recorded after 5 min. Then, 15 μL of a 40 mM NADPH solution was added to each cuvette, and spectral recordings were made every 5 min until a stable maximum peak was achieved (25–30 min). For clarity, only the spectrum at 30 min is presented (spectrum 2). (B) Liver microsomes from a PCN-treated female rat. The same procedure was followed as in (A), except potassium ferricyanide pretreatment was omitted, the protein concentration was 1.35 mg/mL. Note the different absorbance sensitivity scales.

contrast to the results obtained with anti-P-450$_p$, immunoblot analysis of microsomes from TAO-treated rats reacted with anti-P-450$_b$ IgG or anti-P-450$_c$ IgG gave no indication of the presence of P-450$_b$ or P-450$_c$ even though these antigens were readily detected in microsomes from rats treated with phenobarbital or BNF, respectively (results not shown).

Spectral and catalytic characterizations provided further evidence that TAO induced a cytochrome similar to (or identical with) that induced by PCN or glucocorticoids. For example, when microsomes isolated from TAO- or PCN/DEX-treated rats were pretreated with ferricyanide and then incubated with TAO, typical type I difference spectra were observed (Figure 3). Moreover, upon addition of NADPH and further aerobic incubation, the type I spectrum was progressively converted to an absorption peak at 457 nm, indicating enzymatic conversion of the bound TAO to a TAO metabolite which forms the TAO complex (Pessayre et al., 1981). The maximal amounts of TAO complex formed by microsomes from a TAO-treated rat and that formed by microsomes isolated from PCN- or DEX-treated rats were far greater (10–20 times) than the amounts of the TAO complex formed by microsomes prepared from phenobarbital-treated rats or from untreated male rats (Table I). The TAO complex was undetectable in microsomes from an untreated female rat (Table I). These results implicate P-450$_p$ in formation of the TAO complex since immunoreactive P-450$_p$ is modestly in-

Table I: Measurement of TAO Type I Spectrum, TAO Complex Formation in Vitro, and Demethylase Activities in Rat Liver Microsomes[a]

source of microsomes[b]	type I spectrum, $A^{390/420}$	TAO complex, $A^{457/510}$	demethylase activity (nmol mg^{-1} min^{-1})	
			erythromycin	ethylmorphine
UT (M)	0.000	0.006	0.73	9.00
UT (F)	0.000	0.000	0.96	1.07
TAO (M)	0.125	0.175	7.20	11.30
TAO (F)	0.083	0.128	4.50	13.40
PCN (M)	0.027	0.045	2.95	14.50
PCN (F)	0.024	0.056	3.40	12.80
DEX (M)	0.037	0.058	3.09	14.32
DEX (F)	0.040	0.073	4.50	13.64
PB (M)	0.018	0.030	NT[c]	NT

[a] Type I spectrum with TAO, TAO complex formation in vitro, and monooxygenase activity were measured as described in vitro. Spectral results are expressed as OD per milligram of microsomal protein. [b] Liver microsomes were prepared from untreated (UT) male (M) and female (F) rats or from animals treated with TAO, PCN, DEX, or phenobarbital (PB). [c] NT, not tested.

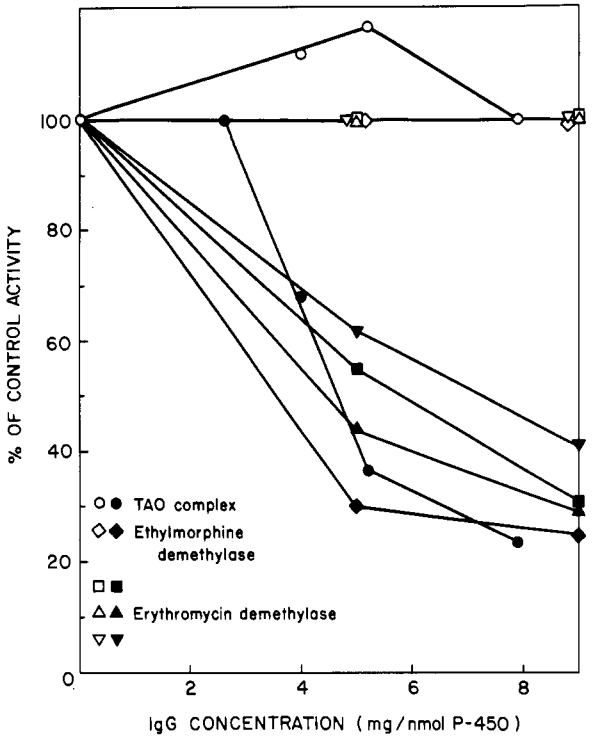

FIGURE 4: Inhibition of anti-P-450$_p$ IgG of liver microsomal monooxygenase activities and TAO complex formation in vitro. Liver microsomes prepared from female rats treated with TAO, PCN, or DEX were preincubated with the indicated amounts of IgG or with buffer (control values), and then monooxygenase activites of TAO complex formation in vitro was determined as indicated under Materials and Methods. Open symbols refer to monooxygenase activity after preincubation with antibodies directed against purified P-450$_b$ or P-450$_c$ whereas closed symbols refer to same activity after incubation with anti P-450$_p$ IgG. The data are given as percent of the control values (no IgG) which were as follows: (○) rate of formation of TAO complex, 0.16 nmol min^{-1} (mg of protein)$^{-1}$; (◇) erythromycin demethylase activity, 4.25 nmol min^{-1} (mg of protein)$^{-1}$; ethylmorphine demethylase activity, 16.7 (□), 2.0 (△), and 2.25 (▽) nmol min^{-1} (mg of protein)$^{-1}$ for microsomes from rats treated with TAO, PCN, and DEX, respectively.

duced by phenobarbital, is present in small amounts in untreated male rats, but is undetectable in untreated females (Figure 2).

It has been shown that erythromycin demethylase activity in rabbit liver microsomes is selectively stimulated by feeding the animals a TAO diet (Bonfils et al., 1983). We found that

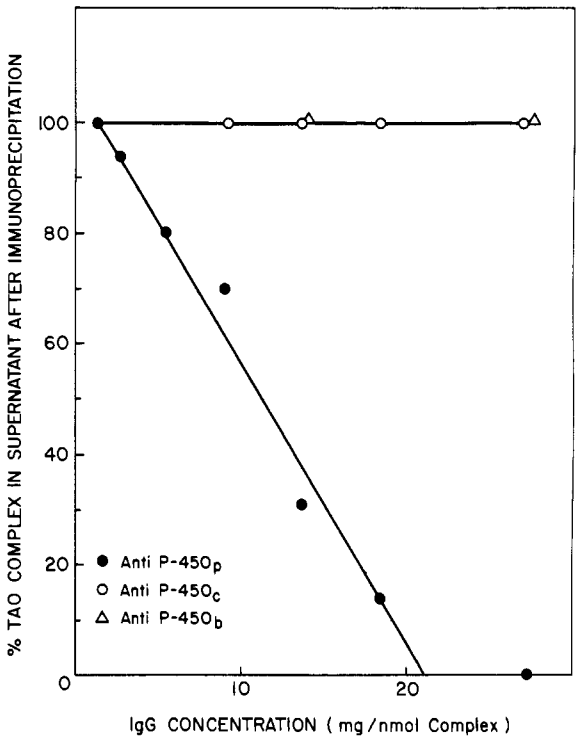

FIGURE 5: Immunoprecipitation by anti-P-450$_p$ IgG of TAO complex formed in vivo. Liver microsomal proteins from a TAO-treated male rat were solubilized, diluted to a concentration of 0.5 nmol of TAO complex/mL, and incubated with varying amounts of antibodies directed against P-450$_p$ (●), P-450$_b$ (△), or P-450$_c$ (○). Immunoreactive proteins were precipitated by addition of *S. aureus* ghosts followed by centrifugation. The amount of TAO complex remaining in the supernatant was quantitated by difference spectroscopy as described under Materials and Methods and was expressed as the percent of the complex initially present in the microsomes.

this enzyme activity was induced in rat liver microsomes prepared from animals treated with TAO or with PCN/DEX (Table I). Similarly, ethylmorphine demethylase activity, which is known to be induced in rat liver microsomes by PCN (Elshourbagy & Guzelian, 1980), also is strongly induced by administration of TAO, especially to female animals.

A final set of experiments with microsomes examined the ability of anti-P-450$_p$ antibodies to specifically inhibit the TAO-induced catalytic activities in microsomes from TAO-treated rats. Additions of increasing amounts of anti-P-450$_p$ IgG proportionally inhibited TAO complex formation (both the rate of its formation and its maximal level) and erythromycin and ethylmorphine demethylase activities (Figure 4). By contrast these activities were not inhibited by nonimmune IgG or by antibodies directed against P-450$_b$ or P-450$_c$. Similar results were obtained with microsomes from DEX-treated rats (data not shown). We also treated microsomes isolated from TAO-treated rats with detergents under conditions that solubilized 100% of the TAO complex initially present in the microsomes. Upon addition of anti-P-450$_p$ IgG the solubilized TAO complex was specifically immunoprecipitated whereas addition of nonimmune IgG, anti-P-450$_b$, or anti-P-450$_c$ IgG were without effect (Figure 5). Since the TAO complex initially present was totally immunoprecipitated by anti-P-450$_p$ IgG, it appears likely that P-450$_p$ accounts for most (if not all) of TAO complex formed in vivo.

Purification of P-450$_{TAO}$ and Comparison with P-450$_p$. To directly examine the properties of the major form of hepatic cytochrome P-450 induced by TAO, we used a procedure that involves solubilization of microsomal cytochromes with cholate followed by chromatography on aminooctylamino-Sepharose

Table II: Amino-Terminal Amino Acid Sequences of Some Liver Cytochromes P-450[a]

cytochrome	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15	homology with P-450$_p$ (%)	reference
P-450$_p$	Met-Asp-Leu-Ile-Phe-Met-Leu-Gln-Thr-Ser-Ser-Leu-Leu-Ala-Ala		see Materials and Methods
P-450$_{TAO}$	Met-Asp-Leu-Ile-Phe-Met-Leu-Gln-Thr-Ser-Ser-Leu-Leu-Ala-Ala	100	
P-450$_b$	Met-Glu-Pro-Ser-Ile-Leu-Leu-Leu-Leu-Ala-Leu-Leu-Val-Gly-Phe	20	Yuan et al. (1983)
P-450$_c$	His*-Ser*-Val-Tyr-Gly-Phe-Pro-Ala-Phe-Thr-Ser-Ala-Thr-Glu-Leu	7	Botelho et al. (1982)
LM3$_c$	Met-Asp-Leu-Ile-Phe-Ser-Leu-Glu-Thr-Trp-Val-Leu-Leu-Ala-Ala	73	Koop et al. (1982)

[a] Asterisk indicates revised sequence (W. Levin, personal communication).

4B and two sequential hydroxylapatite columns. The TAO complex served as a specific marker of the TAO-induced cytochrome in each step of the purification procedure. Although the amount of TAO complex relative to the total cytochrome P-450 concentration gradually decreased during the course of the purification (possibly due to removal of essential stabilizing constituents), 45% of the initial TAO complex appeared to copurify with the final cytochrome P-450$_{TAO}$ fraction. The final preparation (5.5 nmol of CO binding hemoprotein/mg of protein) appeared to be homogeneous when subjected to electrophoresis on SDS–polyacrylamide gels followed by staining with silver (Figure 1). In immunoblots developed with anti-P-450$_p$ IgG, the purified P-450$_{TAO}$ reacted positively and exhibited the same mobility when compared to that of purified P-450$_p$ (12.8 nmol/mg of protein) or of the single immunoreactive band in microsomes isolated from rats treated with TAO or PCN/DEX (Figure 2). In addition, analysis by two-dimensional gel electrophoresis reveals no differences in the mobility of P-450$_{TAO}$ and P-450$_p$ (results not shown).

Cytochrome P-450$_{TAO}$ was found to be low spin in the oxidized state. After treatment with ferricyanide (in order to destroy the remaining TAO complex) the ferrous carbonyl complex exhibited a maximum at 450 ± 0.5 nm. The P-450$_{TAO}$ holocytochrome may be unstable during purification as suggested by the low specific heme content of the purified preparation. Moreover, P-450$_{TAO}$ was rapidly converted to cytochrome P-420 when assayed as dithionite-reduced CO binding hemoprotein. Addition of 0.2% Lubrol PX in the assay buffer doubled the height of the CO peak at 450 nm but did not prevent conversion to cytochrome P-420. Purified P-450$_p$ exhibited similar spectral characteristics (not shown).

In a reconstituted system consisting of purified P-450$_{TAO}$ or P-450$_p$, cytochrome P-450 reductase, phosphatidylcholine, and NADPH, demethylation of ethylmorphine and erythromycin preceded at similar, low rates (4.0 ± 0.5 and 2.1 ± 0.2 nmol of product mg^{-1} min^{-1}, respectively). Even though difference spectroscopy revealed clearly that TAO was bound by the purified cytochromes yielding a type I spectrum, no formation in the TAO complex could be detected (Figure 6). Varying the concentrations of phospholipid (5–200 μg/mL) or Lubrol PX (0.01–0.05%) or addition of purified cytochrome b_5 (equimolar amount with respect to cytochrome P-450) failed to restore the ability of the purified cytochromes to produce the 457-nm-absorbing TAO complex. We have also found that the purified form of rabbit cytochrome P-450 induced by TAO also fails to form the TAO complex in a reconstituted system.[3]

Three final tests revealed no differences between purified P-450$_p$ and P-450$_{TAO}$. First, Ouchterlony double-diffusion

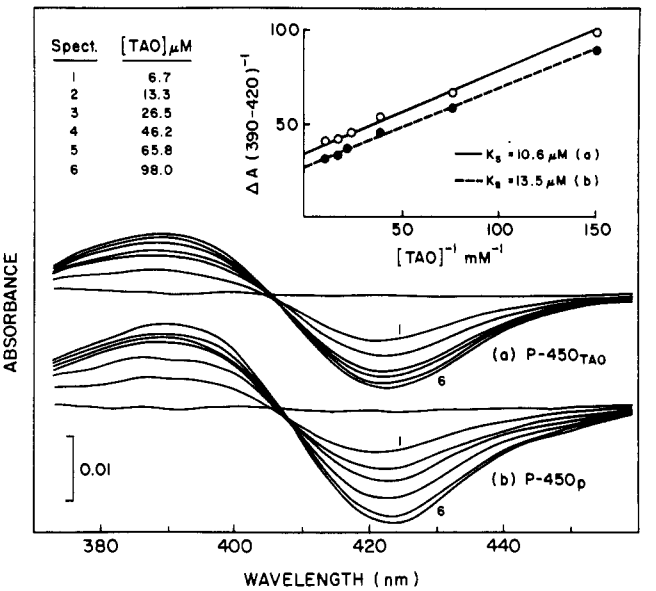

FIGURE 6: Effect of TAO on the spin state of purified P-450$_{TAO}$ and P-450$_p$. P-450$_{TAO}$ or P-450$_p$ were diluted to a final concentration of 0.82 or 1.8 μM of CO binding hemoproteins, respectively, in 0.1 M potassium phosphate buffer containing 1 mM EDTA and 20% glycerol. An equal amount of this solution (750 μL) was placed in both sample and reference cuvettes of an Aminco DW2 spectrophotometer, and base line was recorded. Various amounts of a 5 mM solution of TAO in Me_2SO were then added to the sample cuvette while the same volume of Me_2SO was added to the reference cuvette; 5 min after each addition a difference spectrum was recorded. Spectra 1–6 were obtained with 6.7, 13.3, 26.5, 46.2, 65.8, and 98.0 μM TAO, respectively, taking into account the slight dilution due to additions of the TAO solution. Inset is a double-reciprocal plot of absorbance change between 390 and 420 nm vs. TAO concentration [(a) P-450$_{TAO}$; (b) P-450$_p$].

analyses carried out with anti-P-450$_p$ IgG exhibited a clean continuity of precipitin bands between purified P-450$_p$, P-450$_{TAO}$, and TAO microsomes (not shown). Second, when P-450$_{TAO}$ and P-450$_p$ were digested with either α-chymotrypsin or *S. aureus* V8 and the peptide fragments separated by SDS–polyacrylamide gel electrophoresis, there were no differences between the patterns of proteolytic fragments when the gels were directly silver stained or when the peptides were transferred to nitrocellulose paper and reacted against anti-P-450$_p$ IgG (Figure 7). Finally, automated sequencing of the NH_2-terminal portions of P-450$_{TAO}$ and P-450$_p$ yielded identical results for the first 15 amino acids (Table II). This sequence is clearly divergent from those published for P-450$_b$ (Yuan et al., 1983) or P-450$_c$ (Botelho et al., 1982). We conclude from this series of functional, spectral, biochemical, and immunochemical comparisons of microsomes and of purified hemoproteins that the major form of cytochrome P-450 induced in rat liver by TAO is indistinguishable from P-450$_p$.

[3] P. Maurel et al., unpublished results.

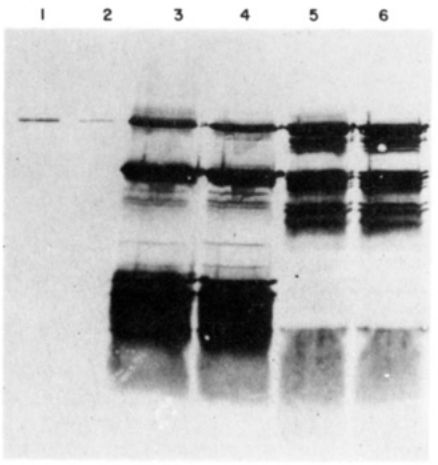

FIGURE 7: Immunoblot of peptide maps of purified P-450$_{TAO}$ and P-450$_p$. Samples of purified P-450 (20 μg of protein) were digested for 30 min with either V8 protease or α-chymotrypsin. Proteolytic digests were resolved by polyacrylamide gel electrophoresis and then analyzed by immunoblotting with anti-P-450$_p$ IgG (see Materials and Methods). Lane 1, P-450$_{TAO}$; lane 2, P-450$_p$; lanes 3 and 4, V8 proteolytic digest of P-450$_{TAO}$ and P-450$_p$, respectively; lanes 5 and 6, α-chymotrypsin digests of P-450$_{TAO}$ and P-450$_p$, respectively.

Discussion

TAO proved to be the most efficacious single inducer of rat liver microsomal cytochrome P-450 yet reported (Guengerich et al., 1982; Heuman et al., 1982). In our rats treated with TAO, the specific content of liver microsomal cytochrome P-450 was usually 25–50% higher than that routinely achieved in this and other laboratories with other effective inducers such as phenobarbital, BNF, or Aroclor 1254. Associated with the increased CO binding hemoprotein in microsomes prepared from TAO treated rats was a complex between the cytochrome and a TAO metabolite with absorbance at 457 nm. This complex is formed in vivo and persists during preparation of liver microsomes and purification of P-450$_{TAO}$. Other forms of cytochrome P-450 catalyze formation of analogous "metabolic intermediate" complexes with metabolites of other substrates such as isosafrole or benzphetamine (Werringloer & Estabrook, 1978; Pershing & Franklin, 1982). It has been argued that metabolic intermediate complexes provide a convenient way to identify, isolate, and purify specific forms of cytochrome P-450 capable of activating such substrates (Fisher et al., 1981; Bornheim & Franklin, 1982), and this proved to be the case for P-450$_{TAO}$. The TAO complex also provided a convenient indicator as a compliment to immunochemical tests for cross-comparisons of purified forms of cytochrome P-450 and for estimations of the amounts of these cytochromes in intact microsomes. The formation of the TAO complex by microsomes from rats treated with DEX implicated P-450$_p$ (known to be induced by glucocorticoid hormones) as the major cytochrome induced by TAO. Indeed, when P-450$_{TAO}$ was isolated, purified, and compared to highly purified P-450$_p$, there were no significant differences recognized by the following tests: (a) mobility of the proteins in one- or two-dimensional SDS–polyacrylamide gel electrophoresis; (b) immunoreactive characteristics based on Ouchterlony double-diffusion analysis or immunoblots; (c) peptide mapping; (d) NH_2-terminal sequence analysis; (e) spectral characteristics; (f) TAO binding properties. These data provide overwhelming evidence that TAO microsomes contain a form of cytochrome P-450 indistinguishable from P-450$_p$.

Evidence of biochemical and immunochemical identity between purified P-450$_{TAO}$ and purified P-450$_p$ is necessary but not sufficient to establish that the major form of cytochrome P-450 induced by TAO is p-450$_p$. For this purpose it was important to analyze microsomes prepared from TAO-treated rats. We demonstrated that TAO treatment selectively caused the appearance of a protein which was identical with P-450$_p$ in its electrophoretic mobility and was specifically recognized by anti-P-450$_p$ antibodies. Moreover, microsomes from TAO-treated rats uniquely catalyzed the conversion of bound TAO to the TAO complex, and this complex, when solubilized, was completely immunoprecipitated by anti-P-450$_p$ antibodies. Finally, preincubation of decomplexed microsomes isolated from TAO-treated rats with anti-P-450$_p$ antibodies blocked the capacity of the microsomes to form the TAO complex and also blocked the induced ethylmorphine or erythromycin demethylase activities. Since all of these characteristics were reproduced in microsomes from PCN/DEX-treated animals, it may be concluded that most (if not all) of the increase in total spectral cytochrome P-450 in animals treated either with TAO or with glucocorticoids can be accounted for by the single cytochrome we have called P-450$_p$.

Amino acid sequence analysis of the NH_2-terminal end of P-450$_p$ revealed no differences through the first 15 residues with that of P-450$_{TAO}$. This sequence shares only three residues (positions 1, 7, and 12) with the published NH_2-terminal sequence of the phenobarbital-inducible cytochrome P-450$_b$ and only one residue (position 11) with BNF inducible P-450$_c$ (Table II). This confirms that even though P-450$_p$ shares regulatory characteristics with P-450$_b$ by being inducible by phenobarbital, P-450$_p$ is a structurally unrelated cytochrome. However, P-450$_p$ bears striking (73%) homology with the NH_2-terminal sequence of rabbit liver LM3c. From this and other characteristics of purified LM3c (Koop et al., 1982) we have proposed that P-450$_p$ and LM3c are homologous forms in these two species.

It is puzzling that neither purified P-450$_{TAO}$ nor purified P-450$_p$ was able to catalyze conversion of TAO to the TAO complex in a reconstituted system in vitro. One possibility may be that other microsomal factors that stabilize the complex are removed during the purification process. Another possibility is that formation of the TAO complex could be a multienzymic process. It has been proposed that formation of the metabolite intermediate complex of benzphetamine, which, like TAO, possesses a primary methylamino group, appears to require first a cytochrome P-450 catalyzed demethylation (Jeffery & Mannering, 1982). This metabolite is then hydroxylalted and converted to a nitrone derivative by a microsomal amine oxidase (Jeffery & Mannering, 1982). The nitrone is then converted to a nitroso derivative that binds tightly to the ferrous iron of the heme prosthetic group of cytochrome P-450, giving a 455-nm-absorbing complex. If the formation of the TAO complex follows a similar path, than it is possible that P-450$_p$ is capable only of forming the N-demethylated derivative of TAO and that microsomal amine oxidase would be required to produce the nitroso derivative ultimately responsible for complex formation.

Recognition of TAO (and presumably other macrolide antibiotics) as inducers of P-450$_p$ provides a set of valuable new compounds to probe the mechanism of induction of this cytochrome. On the basis of a comprehensive study in primary cultures of adult rat hepatocytes of the effects of glucocorticoids, anti-glucocorticoids, and other steroid hormones on de novo synthesis of P-450$_p$, we have proposed that steroid inducers act through a unique stereospecific "receptor-like" mechanism that can be clearly distinguished from the classical

glucocorticoid receptor (Schuetz et al., 1984; Schuetz & Guzelian, 1984a,b). Unanswered is the question of how P-450$_p$ is induced by nonsteroidal compounds, namely, the phenobarbital-like inducers (Guengerich et al., 1982; Heuman et al., 1980; Schuetz & Guzelian, 1984b) and the macrolide antibiotics. It seems improbable that these compounds are direct agonists for the postulated "PCN receptor". It is possible that phenobarbital or TAO induce P-450$_p$ by altering the metabolism of an endogenous steroid inducer. It is also possible that the increased accumulation of cytochrome P-450 in TAO-treated animals is not due exclusively to increased synthesis of P-450$_p$. Preliminary studies suggest that TAO may also prevent the degradation of P-450$_p$ (Watkins et al., 1984).

Interest in the macrolide antibiotics as possible modulators of the cytochrome P-450 system was originally prompted by clinical reports of adverse drug interactions in patients receiving TAO or erythromycin. The mechanism of the toxic interactions remains to be elucidated but appears to involve altered hepatic metabolism of drugs administered simultaneously with macrolide antibiotics (Larrey et al., 1983). Human liver microsomes prepared from biopsies of patients given TAO under controlled conditions have an increased ability to catalytically activate TAO to form the TAO complex (Pessayre et al., 1982). Our present studies have established the equivalence between TAO complex formation and P-450$_p$. Therefore, we predict that a form of cytochrome P-450 homologous to rat P-450$_p$ will be found in human liver.

ACKNOWLEDGMENTS

We thank Dr. F. P. Guengerich for suggesting that P-450$_p$ was induced by TAO and Pamela Barkan for her expert secretarial assistance.

Registry No. TAO, 2751-09-9; P-450, 9035-51-2; ethylmorphine demethylase, 9032-78-4; erythromycin demethylase, 78783-50-3.

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