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Immunochemical Evidence for Two 3-Methylcholanthrene-Inducible Forms of Cytochrome P-448 in Rat Liver Microsomes Using a Double-Antibody Radioimmunoassay Procedure

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SUMMARY

Double-antibody radioimmunoassay (RIA) procedures were developed for the determination of two forms of cytochrome P-448 in crude liver microsomes at the picogram level. The antisera used in the RIAs were produced against two forms of cytochrome P-448 isolated from livers of rats treated with 3,4,5,3′,4′,5′-hexachlorobiphenyl (3,4,5-HCB) and 3-methylcholanthrene (3-MC), referred to as cytochromes P-448$_{HCB}$ and P-448$_{MC}$, respectively. The results obtained with RIA procedures were consistent with results of radial immunodiffusion analysis, sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and estimation of aryl hydrocarbon hydroxylase (AHH) activity. Utilizing the RIAs, both forms of cytochrome P-448 were found in significant quantities in liver microsomes of rats treated with 3-MC, 3,4,5-HCB, isosafrole, and Aroclor 1254 but only in minute concentrations in untreated and phenobarbital-treated rats.

INTRODUCTION

During the past decade RIAs[1] and similar types of techniques (e.g., enzyme-linked immunoadsorbent assay) have been developed and employed for quantitation of a variety of biological and synthetic substances (1). These competitive binding immunoassays provide a combination of sensitivity and performance simplicity unavailable with most other quantitative procedures. The isolation of highly purified hepatic cytochromes P-450 and P-448 from microsomes of chemically treated laboratory animals and the subsequent production of specific antisera against these isoenzymes have allowed for a variety of immunochemical studies. Antisera prepared against specific cytochromes have been used to inhibit differentially the metabolism of xenobiotics in microsomes or reconstituted systems (2, 3) and for histochemical localization and distribution of cytochromes in cells and tissues (4, 5). Recently these antisera have been employed for quantitative determination of specific cytochromes in microsomal preparations of rats treated with various inducers using the techniques of radial immunodiffusion (6) and rocket immunoelectrophoresis (7). In order to circumvent the limitation in sensitivity that is inherent in visual precipitation techniques, the next logical progression in quantitative analysis of cytochrome P-450 was the development of competitive binding immunoassays.

In this study we describe the development of RIAs for quantitation of two forms of hepatic cytochrome P-448, which are referred to as cytochrome P-448$_{MC}$[2] and P-448$_{HCB}$. Cytochrome P-448$_{HCB}$ is a form of cytochrome P-448 recently isolated from livers of rats treated with 3,4,5-HCB. It differs in its molecular weight, chromatographic behavior, substrate specificity, peptide maps, and antigenic characteristics from the major form of cytochrome P-448 (P-448$_{MC}$), isolated from 3-MC treated rats, as well as from cytochrome P-450$_{PB}$, isolated from livers of PB-treated rats (9). The RIAs developed in this study were used to demonstrate for the first time that both forms of cytochrome P-448 are major isozymes induced by 3-MC-type inducers and to quantitate these two forms in livers of control rats as well as rats treated with several inducers.

METHODS

Microsomal preparations. Male Sprague-Dawley rats (100–150 g) were dosed i.p. with PB (75 mg/kg daily for 4 days), 3-MC (50 mg/kg daily for 3 days), or Aroclor 1254 (50 mg/kg daily for 3 days) and killed 24 hr later. 3,4,5-HCB (50 mg/kg) was given as a single dose and the rats were killed 72 hr later. Hepatic microsomes were prepared by differential centrifugation and stored as described earlier (9). Microsomes from at least three identically treated animals were pooled for each RIA experiment. Microsomes were solubilized by resuspen-

[1] The abbreviations used are: RIA, radioimmunoassay; 3,4,5-HCB, 3,4,5,3′,4′,5′-hexachlorobiphenyl; 3-MC, 3-methylcholanthrene; PB, phenobarbital; SDS, sodium dodecyl sulfate; IA, immunoadsorbent; RID, radial immunodiffusion; AHH, aryl hydrocarbon hydroxylase.

[2] Cytochrome P-448$_{MC}$ is presumably identical with the isoenzyme purified by Ryan *et al.* (8) and termed P-450$_C$.

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sion in 0.1 M potassium phosphate buffer (pH 7.4), containing 20% glycerol, 1 mM EDTA, 0.6% cholate, and 0.1% Emulgen and adjusted to a concentration of 1.5 mg of protein per milliliter. The mixture was stirred at room temperature for 30 min and then centrifuged at 150,000 × *g* for 1 hr at 5°. Solubilization of microsomal P-450 from all treatment groups varied from 88% to 100% at this protein concentration.

Isolation of P-448$_{MC}$ and P-448$_{HCB}$. Hepatic cytochrome P-448$_{HCB}$ from 3,4,5-HCB-treated rats and cytochrome P-448$_{MC}$ from 3-MC-treated rats were purified to apparent homogeneity on SDS/polyacrylamide gels as described previously (8). The molecular weight of cytochrome P-448$_{HCB}$ was approximately 52,000 on SDS gels, and the specific content was 18.8 nmoles/mg of protein. The molecular weight of cytochrome P-448$_{MC}$ was approximately 55,000 and the specific content was 13.6 nmoles/mg of protein.

Antiserum. New Zealand White rabbits received intradermal injections in the back of either cytochrome P-448$_{HCB}$ or P-448$_{MC}$ (0.3 mg) emulsified in complete Freund's adjuvant (9). Booster injections, containing 0.2 mg of antigen emulsified in incomplete Freund's adjuvant, were given at 2-week intervals until antisera revealed precipitin bands in Ouchterlony gels. Since antisera of high avidity is desirable for RIA work, antibody titers in immunized rabbits were monitored until a plateau or decay in the titer was observed (approximately 3–6 months). At that time, rabbits were given an additional booster injection consisting of 0.2 mg of antigen in incomplete Freund's adjuvant and bled 10 days later.

Assay buffer. The RIA assay buffer was 0.1 M potassium phosphate to which was added 0.2% Emulgen 911 (Kao-Atlas, Ltd., Tokyo, Japan) and 0.1% gelatin (No. 0143-01; Difco Laboratories, Detroit, Mich.) as a carrier. The buffer was adjusted to pH 7.4 and filter-sterilized.

Preparation of radiolabeled compound. Approximately 10 μg of the purified cytochrome (P-448$_{MC}$ or P-448$_{HCB}$) was radiolabeled with ^{125}I using the *N*-hydroxysuccinimide ester of iodinated *p*-hydroxyphenylpropionic acid (10), commonly referred to as "Bolton-Hunter reagent" (NEX-120; 0.5 mCi; New England Nuclear Corporation, Boston, Mass.) in 50 mM KPO_4 (pH 7.4) containing 20% glycerol. Purified cytochrome was injected into the vial containing the reagent, mixed, and allowed to remain at 4° for 24 hr. The product was then removed and applied to a disposable column (1.5 × 60 cm) of Sephadex G-25 (Pharmacia, Uppsala, Sweden). Radiolabeled material was eluted from the column by gravity using 50 mM KPO_4 (pH 7.4) containing 20% glycerol, 0.25% gelatin, 0.2% emulgen, and 0.02% sodium azide. The column eluant was monitored for radioactivity and the first peak was pooled, aliquoted, and frozen at −70°. Unreacted Bolton-Hunter reagent eluted in a second peak. Over 95% of the radioactivity in the first peak was trichloroacetic acid-precipitable and had an estimated specific activity of approximately 37–39 μCi/μg. Attempts to radioiodinate cytochrome P-448$_{HCB}$ by the chloramine T method (11) or enzymatically with lactoperoxidase (12) resulted in considerably less radioactivity bound to protein (<5%).

RIA procedure. RIAs require some means of separating antibody-bound material from nonbound material. The method used in the present study involved precipitating the antibody-cytochrome complex with an optimal dilution of goat antibodies directed against rabbit γ-globulin. Details of "double antibody" assays have been described by Hunter (13). In the RIA for cytochrome(s) P-450, unlabeled cytochrome competes with ^{125}I-labeled cytochrome for the binding antibody. The decrease in precipitable radioactivity is a measure of the amount of unlabeled cytochrome in the test sample.

In order to minimize potential cytochrome denaturation, all buffers and assay tubes were placed on ice or at 4°, whenever possible. Test samples (unknown or purified standards) were diluted in assay buffer, and 0.2-ml aliquots of various dilutions were added to glass tubes (12 × 75 mm). This was followed by the addition of 0.2 ml of P-448$_{MC}$ or P-448$_{HCB}$ immune sera appropriately diluted in assay buffer. A dilution of antiserum that bound about 40% of the radiotracer dose (~1:30,000) was added to the tubes. This was followed by the addition of approximately 4,000 cpm (8,000 dpm) of the corresponding ^{125}I-labeled form of cytochrome diluted in assay buffer (~4,000 cpm/40 μl). Following overnight incubation at 4°, 40 μl of assay buffer containing 0.1% rabbit γ-globulin (Miles Laboratories, Inc., Elkhart, Ind.) were added to each tube followed by 0.2 ml of goat anti-rabbit γ-globulin (1.25 units; Calbiochem, San Diego, Calif.) in assay buffer containing 0.05 mM EDTA. The tubes were incubated for an additional 4 hr at 4° and centrifuged (500 × *g*, 30 min, 4°), and the radioactivity in the precipitate was determined in a γ counter (PRIAS, Packard AutoGamma scintillation spectrometer).

The blank received radiotracer, assay buffer, and anti-rabbit globulin. The zero control (B_0) received diluted antiserum in addition to the radiotracer, assay buffer, and anti-rabbit γ-globulin but no unlabeled cytochromes. The remaining tubes received antiserum, radiotracer, rabbit γ-globulin, and either purified standards, diluted microsomal preparations, or cytochromes being evaluated for cross-reactivity (B). All assays and blanks were run in triplicate.

A calibration curve using known standards was obtained as follows using mean values of replicate counts. First, all counts were corrected by subtracting counts per minute in the precipitate of the blank tubes. Percentage inhibition was obtained from the equation $(B/B_0 - 1) \times 100$. A straight-line calibration curve was prepared, plotting the above equation for the standards versus nanograms of purified cytochrome added to each tube using linear-log paper. The linear portion of this curve was used to determine the amount of cross-reactivity or inhibition by various samples.

Antiserum adsorptions. To minimize cross-reacting antibodies, solid-phase IAs were prepared. An IA for purification of P-448$_{MC}$ immune sera was prepared by coupling approximately 20 mg of partially purified cytochrome P-450$_{PB}$ to 3.0 g of CNBr-activated Sepharose 4B (Sigma Chemical Company, St. Louis, Mo.). Cross-reactive antibodies to P-450$_{PB}$ were removed from P-448$_{MC}$ immune sera by multiple passages (4.0-ml aliquots) over the IA. The cross-reactive antibodies were eluted with 2 M sodium thiocyanate in NaCl-Tris buffer (0.14 M NaCl,

0.01 M Tris-HCl, pH 7.4). Potential cross-reactive antibodies to cytochrome P-450$_{MC}$ were removed from P-448$_{HCB}$ immune sera by passage over a solid support containing covalently bound cytochrome P-448$_{MC}$.

Gel diffusion. Ouchterlony double-diffusion was performed in assay buffer containing 0.9% agarose (Sigma Chemical Company). Cytochrome concentrations in solubilized hepatic microsomes were quantitated by RID analysis (14). The agarose gel was composed of assay buffer, 0.9% agarose, and a 1:15 dilution of the appropriate antisera (added in a 56° water bath). Plugs (3 mm) were removed from the agarose gel and replaced with 15 μl of duplicate dilutions of either purified cytochrome standards (8–64 μg/ml) or microsomal preparations (test samples). The RID plates were allowed to incubate at 4° for 24 hr, washed extensively with saline, and stained with a diluted solution of protein stain (0.5% amido schwartz 10B, 5% mercuric chloride (w/v), and 5% glacial acetic acetic) until precipitation rings were easily visualized. Ring diameters were determined with use of a hand-held micrometer.

Other methods. The cytochrome P-450 content was determined from the CO-reduced difference spectrum by the method of Omura and Sato (15). AHH activity was measured at 37° by estimation of fluorescent metabolites as previously described (16). Protein was determined by the method of Lowry *et al.* (17), using bovine serum albumin as a standard. SDS/polyacrylamide gel electrophoresis was performed as described by Laemmli (18).

RESULTS AND DISCUSSION

The final assay conditions, including buffer pH, buffer ionic strength, time of incubation, and detergent concentrations, were found to be optimal for both the cytochrome P-448$_{MC}$ and P-448$_{HCB}$ RIA systems. While a variety of ionic and nonionic detergents were tested in the RIA to help eliminate some of the relative insolubility of cytochrome P-448$_{HCB}$ in physiological buffer, we found that 0.2% Emulgen 911 had a minimal effect on antibody-antigen reactions while preventing nonspecific precipitation. The use of Emulgen 911 in other types of immunological assays for cytochromes has been described (6).

Cross-reactivity to cytochrome P-450$_{PB}$ antigen was readily evident in antiserum produced against P-488$_{MC}$, being detectable not only by RIA (30% cross-reactivity) but in Ouchterlony gels as well (Table 1). The precipitin band was not present in Ouchterlony gels when the antiserum was adsorbed with cytochrome P-450$_{PB}$ covalently bound to a solid support IA and is thus similar to earlier studies by Thomas, *et al.* (6) in which P-450$_{PB}$ cross-reactivity was also found in P-448$_{MC}$ immune sera and removed by similar procedures. P-450$_{PB}$ cross-reactivity was reduced to 8% in the absorbed antisera as determined by RIA. This suggested that some of the antibody activity was directed against shared antigenic determinants between cytochrome P-450$_{PB}$ and P-448$_{MC}$ or that one of the cytochromes is contaminated with a small amount of the other isozyme. The remaining cross-reacting antibodies (8%) may suggest the second alternative. The 8% remaining cross-reactivity should be within acceptable limits for most uses of the RIA. Antiserum to cytochrome P-488$_{MC}$ did not cross-react with cytochrome P-448$_{HCB}$ at any concentration tested.

TABLE 1

Cross-reactivity of P-448$_{MC}$ and P-448$_{HCB}$ immune sera in the RIA and Ouchterlony gels prior to and following passage over immunoadsorbents

Cytochrome	% Cross-reactivity[a] of P-448$_{MC}$ antiserum (band formation)		% Cross-reactivity of P-448$_{HCB}$ Antiserum (band formation)	
	Pre-IA	Post-IA[b]	Pre-IA	Post-IA[b]
P-448$_{HCB}$	0 (−)[c]	0 (−)	100[d] (+)	100[d] (+)
P-448$_{MC}$	100[d] (+)	100[d] (−)	5 (±)	0 (−)
P-450$_{PB}$	30 (+)	8 (−)	0 (−)	0 (−)

[a] Percentage cross-reactivity = 100 × (Inhibition of ^{125}I-binding by *X* pmoles of test compound/inhibition by *X* pmoles of purified P-448 (MC or HCB) when *X* = 20 pmoles (1 μg) in the P-448$_{HCB}$ RIA and 0.2 pmole (0.1 μg) in the P-448$_{MC}$ RIA.

[b] P-448$_{MC}$ immune sera were passed through an IA coupled with semipurified P-450$_{PB}$ while P-448$_{HCB}$ antisera were passed through on IA containing semipurified P-448$_{MC}$.

[c] −, No precipitin band present in Ouchterlony gel; ±, weak precipitin band present; +, easily observed precipitin band.

[d] Value set at 100%.

Antiserum produced following immunization with cytochrome P-488$_{HCB}$ did not contain the degree of cross-reactivity found in P-448$_{MC}$ antiserum. While cross-reactivity was not observed between the antisera and cytochrome P-450$_{PB}$ at the concentrations tested, some cross-reactivity was present against P-448$_{MC}$ (≤5.0%). A weak precipitation band was sometimes visible between P-448$_{HCB}$ immune sera and P-448$_{MC}$ antigen (8). Cross-reactivity to P-448$_{MC}$ was completely removed following passage of the antiserum through a solid-support IA containing covalently bound P-448$_{MC}$ as determined by RIA and Ouchterlony diffusion with little, if any, loss of antibody activity against P-448$_{HCB}$ (Table 1).

A typical standard curve for inhibition of the binding of radioiodinated cytochromes P-448$_{MC}$ and P-448$_{HCB}$ to their corresponding antisera is shown in Fig. 1. The range of detection for the RIA to P-448$_{HCB}$ is approximately 1–100 pmoles, whereas in the P-448$_{MC}$ RIA the detection limit is in the order of 0.05–10 pmoles using these antisera. These values are based on nonoverlapping ranges of blank and test replicates. Furthermore, there was no interference from test samples. Standard curves for cytochrome P-448$_{MC}$ analyzed in the presence of solubilized control microsomal P-450 were identical with curves prepared in the absence of solubilized microsomes. Although the limit of sensitivity was greater for cytochrome P-448$_{MC}$ than for P-448$_{HCB}$, the linearity of the P-448$_{MC}$ curve decreased when approaching higher levels of cytochrome. Therefore, unknown samples should be tested at several dilutions and values determined in a linear portion of the curve.

The concentration of cytochromes P-448$_{MC}$ and P-448$_{HCB}$ in liver microsomes from control and chemically induced rats is presented in Table 2. Approximately 80% of the total cytochrome P-450 could be identified in 3-MC-treated rats, with cytochromes P-448$_{MC}$ and P-448$_{HCB}$ accounting for 50% and 30% of the total cytochrome P-450, respectively. In contrast to 3-MC treatment, the principal form of cytochrome P-450 in liver microsomes of 3,4,5-HCB-treated rats is P-448$_{HCB}$ (approximately 35% of total P-450), although a considerable

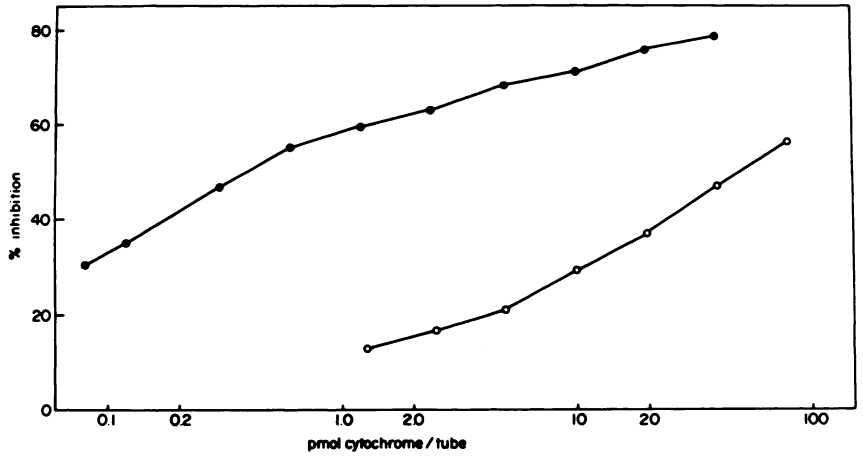

FIG. 1. *Standard curve*
Determination of cytochromes P-448$_{MC}$ (●) and P-448$_{HCB}$ (○) using their respective antisera.

TABLE 2

Concentrations of cytochrome P-448$_{MC}$ and P-448$_{HCB}$ in liver microsomes from chemically treated rats as determined by RIA and RID

Chemical treatment	Total P-450[a]	Specific isoenzyme content[b]			
		P-448$_{MC}$		P-448$_{HCB}$	
		RIA	RID	RIA	RID
	nmoles/mg protein	*nmoles/mg (%)*	*nmoles/mg (%)*	*nmoles/mg (%)*	*nmoles/mg (%)*
None	0.71	<0.01 (<1.4)	ND[c] (–)	0.01 (1.4)	ND (–)
PB	1.56	0.01 (0.6)	ND (–)	0.06 (3.8)	ND (–)
Aroclor	1.38	0.22 (16)	0.28 (20)	0.19 (14)	0.23 (17)
3-MC	2.00	0.96 (48)	1.00 (50)	0.58 (29)	0.68 (34)
3,4,5-HCB	2.96	0.68 (23)	0.83 (28)	1.09 (37)	1.33 (45)
Isosafrole	1.09	0.11 (10)	—[d]	0.43 (40)	—[d]

[a] Total cytochrome P-450 content was determined spectrally as described under Methods.

[b] Values for specific isozymes were determined by RIA and confirmed by RID on the same microsomal preparations. Each determination was made on a microsomal pool from at least three animals. RIA determinations were performed in triplicate at several dilutions in at least two separate experiments. Values in parentheses represent the percentage of total cytochrome P-450 represented by each isozyme determined by the formula (nmoles isozyme/mg microsomal protein)/(total cytochrome P-450/mg microsomal protein) × 100.

[c] ND, Not determined, since precipitin ring was too small to measure accurately.

[d] Not determined.

quantity of cytochrome P-448$_{MC}$ (25%) was found. Cytochromes P-448$_{MC}$ and P-448$_{HCB}$ each constituted approximately 15% of the total cytochrome P-450 content in liver microsomes of Aroclor 1254-treated rats. Aroclor 1254 is a mixture of polychlorinated biphenyls which contains both 3-MC- and PB-type inducers (19). It should be noted that the dose used produces greater induction in females (19) and that a larger dose would probably be more effective in males. These results indicate that 3-MC, 3,4,5-HCB, and Aroclor 1254 exposure can induce at least two major forms of cytochrome P-448 in the livers of male rats. Neither of these forms of cytochrome was present in appreciable amounts in PB-treated or untreated rats.

Quantitative values obtained by RIA and RID for the two forms of cytochrome P-448 in rat liver microsomes were in good agreement, although slightly higher values were obtained for cytochrome P-448$_{HCB}$ by RID than RIA (Table 2). We have noted that P-448$_{HCB}$ is less soluble in physiological buffers than are other forms of cytochrome P-450. Thus, it would appear that a small but appreciable amount of the cytochrome is nonspecifically precipitated during incubation, leading to slightly lower values in the RIA. This is evident from the RIA in "blank" control tubes (lacking antibody) in which 10% of the total radiolabeled P-448$_{HCB}$ will nonspecifically precipitate in the RIA as compared with less than 3% of the radiolabeled P-448$_{MC}$. Of the two techniques, RIA has a greater potential for detecting cross-reacting antibodies as well as observing contaminating forms of cytochrome, as would be expected since it has a sensitivity approximately 2–3 logs greater than RID.

Although 3,4,5-HCB has been classified as a 3-MC inducer (19), only one form of cytochrome P-448 has been isolated from 3-MC-induced rats (8). However, 3-

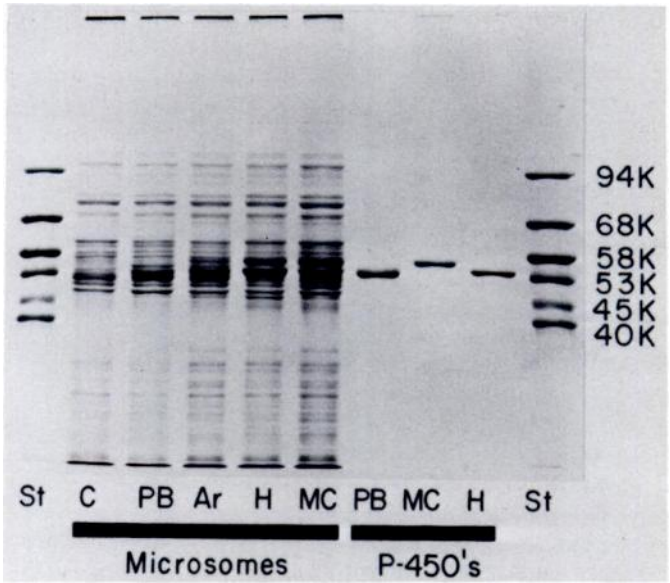

FIG. 2. *SDS/polyacrylamide gel of microsomes and purified cytochromes from PB-, Aroclor 1254-, 3,4,5-HCB-, and 3-MC-treated rats*
St, Standards with the indicated molecular weights. Electrophoresis of microsomes (*M*) (15 μg of protein) from control (*C*), PB-, Aroclor 1254 (*Ar*)-, 3,4,5-HCB (*H*)-, or 3-MC (*MC*)-treated rats or purified cytochrome P-450 (*P-450's*) (2 μg of each): cytochrome P-450$_{PB}$, P-448$_{MC}$, and cytochrome P-448$_{HCB}$ (*H*) were carried out in those wells indicated.

MC-type inducers induce two major forms of cytochrome P-448 in two other species, rabbits and mice (20, 21). Recent work from this laboratory demonstrated that 3,4,5-HCB induces a second form of cytochrome P-448 which differs from the form purified from 3-MC-induced rats in molecular weight, substrate specificity, antigenic characteristics, oxidized spectra, and peptide maps (9). The present study shows for the first time that two forms of cytochrome P-448 are induced by both 3-MC and 3,4,5-HCB, and that both isozymes represent a large proportion of the total cytochrome in induced animals. In contrast, constitutive levels of these isozymes are very low. The molecular weight and spectral characteristics of cytochrome P-448_{HCB} are similar to those reported for a cytochrome isolated from isosafrole-treated rats (P-450_d) (22). RIA data indicated that cytochrome P-448_{HCB} is indeed increased in isosafrole-treated rats, suggesting that these cytochromes may be identical. Isosafrole also produced a much smaller increase in P-448_{MC}. Figure 2 shows the results of an SDS/polyacrylamide gel electrophoresis of liver microsomes from control, 3-MC-, 3,4,5-HCB-, PB-, and Aroclor-treated rats. Two protein-staining bands with molecular weights corresponding to those of cytochromes P-448_{HCB} (52K) and P-448_{MC} (*55K*) were present in 3-MC- and 3,4,5-HCB induced rat microsomes. Furthermore, 3,4,5-HCB produced a larger increase in the 52K molecular weight band than 3-MC. Smaller increases in these two bands were apparent in Aroclor 1254-treated rats.

The present study indicates that cytochrome P-448_{MC} represents a lower percentage of total cytochrome P-450 in 3-MC-treated male rats (50%) than the 70–80% reported by Thomas *et al.* (23) using RID assays and by Pickett *et al.* (7) using a rocket immunoelectrophoresis assay. However, the existence of a second form of cytochrome P-448 in rat liver microsomes was not recognized at the time of the early studies. Therefore, the antibodies used in earlier studies may have been cross-reactive with cytochrome P-448_{HCB}. Moreover, SDS/polyacrylamide gel electrophoresis of liver microsomes from 3-MC-induced rats from our laboratory are consistent with the results of RIA and RID assays. The gels indicate the induction of approximately equivalent amounts of the 52,000 and 55,000 molecular weight forms of cytochrome P-448 by 3-MC. Although quantitative results are not obtained by this method, electrophoresis of purified cytochromes P-448_{HCB} and P-448_{MC} indicates that equivalent amounts of protein do appear comparable visually on these gels (Fig. 2).

In a reconstituted monooxygenase system, purified cytochrome P-448_{MC} metabolizes benzo[*a*]pyrene (AHH activity) with a turnover number of 14 nmoles/min/nmole of P-448_{MC}, while cytochrome P-448_{HCB} has no appreciable activity (<0.1 nmole/min/nmole of P-448_{HCB}) toward this substrate (9). Although the forms of cytochrome P-450 which metabolize benzo[*a*]pyrene in control microsomes have not been identified, AHH activity in microsomes from 3-MC- and 3,4,5-HCB treated rats may be an index of the cytochrome P-448_{MC} content. Table 3 indicates that Aroclor, 3,4,5-HCB, and 3-MC increase AHH activity 8-, 12-, and 27-fold, respectively. The increases in cytochrome P-448_{MC} content as determined by RIA (22-, 68- and 96-fold) are larger than the increases in AHH, presumably because benzo[*a*]pyrene is metabolized by other forms of cytochrome P-450 in control microsomes. However, the relative magnitude of the increases produced by Aroclor, 3,4,5-HCB, and 3-MC are consistent with the RIA data.

TABLE 3

AHH enzyme activity in liver microsomes of chemically treated rats

Each value is the average of duplicate analyses of pooled microsomes from at least three identically treated animals.

Treatment	AHH activity	Increase
	nmoles/mg microsomal protein/min	%
Control	0.41	—
PB	0.93	2×
Aroclor 1254	3.35	8×
3,4,5-HCB	5.12	12×
3-MC	11.18	27×

The development and validation of a sensitive double-antibody RIA procedure for quantitative analysis of these forms of cytochrome in crude microsomal preparations are described. The sensitivity of this assay could be increased further by increasing the specific activity of the radioiodinated cytochromes or by improvement of antisera, possibly through production of monoclonals. This assay has been used to demonstrate that two major forms of cytochrome P-448 are induced in rats by 3-MC-type inducers. Neither of these forms is induced by PB, and constitutive levels of these isozymes are very low.

Note added in proof. A more recently radiolabeled preparation of cytochrome P-448_{HCB} has resulted in a more sensitive standard curve for the RIA for P-448_{HCB} with a range of detection similar to that for P-448_{MC} (0.1–10 pmoles/assay), presumably because less protein was denatured during the second iodination.

REFERENCES

1. Van Vunakin, H., and J. L. Langone (eds.). Immunochemical techniques. *Methods Enzymol.* **84D**:607–656 (1981).
2. Thomas, P. E., A. Y. H. Lu, D. Ryan, S. B. West, J. Kawalek, and W. Levin. Immunochemical evidence for six forms of rat liver cytochrome P450 obtained using antibodies against purified rat liver cytochromes P450 and P448. *Mol. Pharmacol.* **12**:746–758 (1976).
3. Thomas, P. E., A. Y. H. Lu, S. B. West, D. Ryan, G. T. Miwa, and W. Levin. Accessibility of cytochrome P450 in microsomal membranes: inhibition of metabolism by antibodies to cytochrome P450. *Mol. Pharmacol.* **13**:819–831 (1977).
4. Baron, J., J. A. Redick, and F. P. Guengerich. An immunohistochemical study of the localizations and distributions of phenobarbital- and 3-methylcholanthrene-inducible cytochromes P-450 within the livers of untreated rats. *J. Biol. Chem.* **256**:5931–5937 (1981).
5. Serabit-Singh, C., C. R. Wolf, R. M. Philpot, and C. G. Plopper. Cytochrome P-450: localization in rabbit lung. *Science (Wash. D. C.)* **207**:1469–1473 (1980).
6. Thomas, P. E., D. Korzeniowski, D. Ryan, and W. Levin. Preparation of monospecific antibodies against two forms of rat liver cytochrome P-450 and quantitation of these antigens in microsomes. *Arch. Biochem. Biophys.* **192**:524–532 (1979).
7. Pickett, C. B., R. L. Jeter, J. Morin, and A. Y. H. Lu. Electrochemical quantitation of cytochrome P-450, cytochrome P-448, and epoxide hydrolase in rat liver microsomes. *J. Biol. Chem.* **256**:8815–8820 (1981).
8. Ryan, D. E., P. E. Thomas, D. Karzenlowski, and W. Levin. Separation and characterization of highly purified forms of liver microsomal cytochrome P-450 from rats treated with polychlorinated biphenyls, phenobarbital, and 3-methylcholanthrene. *J. Biol. Chem.* **254**:1365–1374 (1979).
9. Goldstein, J. A., P. Linko, M. I. Luster, and D. W. Sundheimer. Purification and characterization of a second form of hepatic cytochrome P-448 from rats treated with a pure polychlorinated biphenyl isomer. *J. Biol. Chem.* **257**:2702–2707 (1982).

10. Rudinger, J., and U. Ruegg. Preparation of *N*-succinimidyl-3-(4-hydroxyphenyl)proprionate. *Biochem. J.* **133**:538–539 (1973).
11. McConahey, P. J., and F. J. Dixon. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy* **29**:185–187 (1966).
12. Marchalonis, J. J. An enzymic method for the trace iodination of immunoglobulins and other proteins. *Biochem. J.* **113**:299–305 (1969).
13. Hunter, W. M. Radioimmunoassay, in *Handbook of Experimental Immunology Radioimmunoassays* (D. M. Weir, ed.). Blackwell Scientific; Oxford, England, 17.1–17.36 (1973).
14. Mancini, G., A. D. Carbonara, and J. E. Heremans. Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* **2**:235–246 (1965).
15. Omura, T., and R. Sato. The carbon-monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties. *J. Biol. Chem.* **239**:2379–2385 (1964).
16. Nebert, D. W., and H. V. Gelboin. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. II. Cellular responses during enzyme induction. *J. Biol. Chem.* **243**:6242–6249 (1968).
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. R. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
18. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* (*Lond.*) **277**:680–685 (1970).
19. Goldstein, J. A., P. Hickman, H. Bergman, J. D. McKinney, and M. P. Walker. Separation of pure polychlorinated biphenyl isomers into two types of inducers on the basis of induction of cytochrome P-450 or P-448. *Chem. Biol. Interact.* **17**:69–87 (1977).
20. Johnson, E. F., and U. Müller-Eberhard. Resolution of two forms of cytochrome P-450 from liver microsomes of rabbits treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J. Biol. Chem.* **252**:2839–2845 (1977).
21. Negishi, M., and D. W. Nebert. Structural gene products of the Ah locus: genetic and immunochemical evidence for two forms of mouse liver cytochrome P-450 induced by 3-methylcholanthrene. *J. Biol. Chem.* **254**:11015–11023 (1979).
22. Ryan, D. E., P. E. Thomas, and W. Levin. Hepatic microsomal cytochrome P-450 in rats treated with isosafrole. *J. Biol. Chem.* **255**:7941–7955 (1980).
23. Thomas, P. E., L. Reik, D. E. Ryan, and W. Levin. Regulation of three forms of cytochrome P-450 and epoxide hydrolase in rat liver microsomes: effects of age, sex and induction. *J. Biol. Chem.* **256**:1044–1052 (1981).

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