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Pulmonary Cytochromes P-450 from Rabbits Treated with 3-Methylcholanthrene

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SUMMARY

Pretreatment of rabbits with 3-methylcholanthrene caused a consistent increase in total cytochrome P-450 content and benzo[*a*]pyrene hydroxylase activity, but no change in ethylmorphine *N*-demethylase activity in lung microsomes. In addition, the CO-difference spectrum of the reduced microsomes showed a shift in the Soret maximum to a shorter wavelength. A slight increase in the ratio 455:430 nm peaks in the ethyl isocyanide difference spectrum of pulmonary microsomes was also observed. Column chromatographic separation of pulmonary cytochromes P-450 demonstrated that 3-methylcholanthrene had no significant effect on cytochromes P-450$_{I}$ and P-450$_{II}$. However, the polycyclic aromatic hydrocarbon induced a new form of this hemeprotein, designated as cytochrome P-450$_{III}$. CO-difference spectra of cytochromes P-450$_{I}$, P-450$_{II}$, and P-450$_{III}$ showed absorption maxima at 452, 450 and 448.4 nm, respectively. In addition, the ethyl isocyanide difference spectral properties of cytochrome P-450$_{III}$ were different from the other two cytochromes. At room temperature, cytochromes P-450$_{I}$ and P-450$_{III}$ had faster reduction rates and were less stable than P-450$_{II}$. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that cytochrome P-450$_{III}$ had a slower electrophoretic mobility than cytochromes P-450$_{I}$ and P-450$_{II}$. Cytochromes P-450$_{I}$ and P-450$_{III}$ possessed less heme-associated peroxidase activity than P-450$_{II}$. These studies demonstrate that 3-methylcholanthrene has the ability to induce a new and distinct form of cytochrome P-450 in rabbit lung.

INTRODUCTION

Humans may be exposed to xenobiotics through inhalation. Lung contains a cytochrome P-450-dependent mixed-function oxidase system which has the ability metabolically to detoxify or activate such foreign chemicals. This system consists of multiple forms of cytochrome P-450, NADPH-cytochrome *c* (P-450) reductase, and phospholipid (1–4). In rabbit lung, two terminal oxidases, cytochromes P-450$_{I}$ and P-450$_{II}$, have been isolated and have been shown to differ in catalytic, electrophoretic, structural, and immunological properties (5–7). In reconstituted systems, the pulmonary enzymes are able to catalyze the oxidations of a number of exogenous substrates, including drugs, carcinogens, and pulmonary toxins (3–5). In this respect, rabbit lung cytochromes P-450 are similar to liver cytochromes P-450 isolated from rabbits and other species (8). However, the pulmonary monooxygenases are markedly different from the hepatic enzymes in responses to several powerful inducers such as phenobarbital, TCDD,[1] and the PCBs mixture, Aroclor 1254. Treatment of rabbits with phenobarbital had no significant effect in lung (9, 10) despite the observation that pulmonary cytochrome P-450$_{I}$ is identical with the hepatic phenobarbital-inducible cytochrome P-450 (7, 10). Treatment with TCDD did not cause any change in the total cytochrome P-450 content, and yet a new form of cytochrome P-450 was induced in lung (11). In marked contrast, PCBs caused a significant decrease in lung microsomal cytochrome P-450 content (12). In this regard, PCBs caused a selective decrease in cytochrome P-450$_{I}$, and no effect on cytochrome P-450$_{II}$, contents of rabbit lung (13).

3-MC is a potent inducer of aryl hydrocarbon hydroxylase activity in rats. It is widely used as a prototypic inducing agent, since in rats it is a powerful inducer of cytochrome P-448 and aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase activity in liver, lung, and other tissues. The effects of 3-MC on benzo[*a*]pyrene metabolism has also been studied in lungs of many other experimental animals (14). Little or no increase in total cytochrome P-450 content was observed in lungs of 3-MC-treated rats (12, 15), mice (16), hamsters (17), and rabbits (12, 18–20).

In the present studies, we have examined the effects of pretreatment of rabbits with 3-MC on the catalytic and spectral properties of lung microsomes. In addition, the

[1] The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCBs, polychlorinated biphenyls; 3-MC, 3-methylcholanthrene; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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chromatographic, spectral, and electrophoretic properties of partially purified pulmonary cytochromes P-450 were compared using untreated and 3-MC-treated rabbits. The results of these studies demonstrate that, although *total* cytochrome P-450 content was minimally affected, 3-MC caused the induction of a hemeprotein, designated cytochrome P-450$_{III}$, which was not detectable in microsomes from lungs of untreated rabbits.

MATERIALS AND METHODS

Treatment of rabbits. Male New Zealand White rabbits weighing 2.7–3.9 kg were used. 3-MC was dissolved in corn oil and administered i.p. to rabbits at a dose of 25 mg/kg/day for 4 days, and the animals were killed on day 5. Control rabbits received corn oil only. Lungs were removed and homogenized, and microsomes were prepared as described previously (12).

Analytical procedures. Cytochrome P-450 content was determined by the method of Omura and Sato (21), using an Aminco DW-2a spectrophotometer equipped with microprocessor data analyzer. Because of contamination with hemoglobin, cytochrome P-450 in rabbit lung microsomes was quantified by determining the dithionite-induced difference spectrum (22). Benzo[*a*]pyrene hydroxylase activity was determined as described previously (12, 23). Ethylmorphine *N*-demethylase activity was determined as described by Alvares and Mannering (24), with the following modifications. Nicotinamide was excluded from the cofactor mixture, and microsomal protein content was increased to 5 mg. Reaction rates were linear under these conditions. The ethyl isocyanide-induced difference spectrum was determined as described previously (25) except that the concentration of ethyl isocyanide added to the sample cuvette was 0.4 mM, in order to obtain maximal peak heights. NADPH-cytochrome *c* reductase activity was determined by the method of Phillips and Langdon (26). The cytochrome b_5 content in the column eluates was determined from the absolute spectra of the oxidized and reduced forms of the hemeprotein (27). Protein concentrations of the microsomal suspensions were determined by the method of Lowry *et al.* (28). Partially purified cytochrome P-450 preparations were dialyzed against 10 mM potassium phosphate buffer (pH 7.4) overnight and then analyzed for protein content by the modification (29) of the method of Lowry *et al.* (28).

Preparation of partially purified cytochromes P-450$_I$, P-450$_{II}$, and P-450$_{III}$. Purification of the cytochromes was carried out essentially by the method of Philpot and his associates (4, 6, 7). The microsomes were solubilized as described previously (12, 13). The solubilized microsomes were applied to a DEAE-cellulose (Whatman DE-52) column (2.5 × 20 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.7), 0.1 mM dithiothreitol, 0.1 mM EDTA, and 20% glycerol (Buffer A) containing 0.1% sodium cholate. Cytochrome P-450$_I$ was eluted from the column using 10 mM Buffer A containing 0.1% sodium cholate and 0.2% Emulgen 911. Following the elution of P-450$_I$, the column was eluted with a linear KCl gradient, 0–0.5 M KCl, using 500 ml of 10 mM Buffer A containing 0.1% sodium cholate and 0.2% Emulgen 911. This gradient elution effectively separated cytochrome P-450$_{III}$ from cytochrome P-450$_{II}$, and the two cytochromes were eluted from the column in different fractions. The following procedure was carried out to remove Emulgen 911, the nonionic detergent, from cytochromes. Fractions from the DE-52 column that contained cytochrome P-450$_I$ were combined and diluted 5-fold with 10 mM Buffer A. The diluted sample was applied to a hydroxylapatite column equilibrated with 10 mM Buffer A, as described by Wolf *et al.* (10). The column was washed with 10 mM Buffer A and then with 100 mM Buffer A. Cytochrome P-450$_I$ was then eluted from the column using 200 mM Buffer A containing 0.2% sodium cholate. Fractions from DE-52 column that contained cytochrome P-450$_{II}$ or P-450$_{III}$ combined and concentrated by ultrafiltration using an Amicon PM-30 membrane. The concentrated sample, 5–10 ml, was applied to a Porapak Q column (1.6 × 10 cm) previously equilibrated with 10 mM Buffer A, as described by Slaughter *et al.* (7). Cytochrome P-450$_{II}$ or P-450$_{III}$ was eluted from the column using the 10 mM Buffer A. The above procedures reduced the nonionic detergent concentrations in the hemeproteins to less than 0.07%.

Gel electrophoresis of cytochromes P-450$_I$, P-450$_{II}$, and P-450$_{III}$. Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate was conducted using the discontinuous system of Laemmli (30). The stacking gel contained 3% acrylamide, and the separation gel (11 × 8.2 × 0.27 cm) contained 10% acrylamide. Electrophoresis was carried out at 7° at 10 and 20 mamp/gel during stacking and separation, respectively, until the tracking dye, bromphenol blue, reached the bottom of the slab. The electrophoretic separation required about 7 hr. The gel was stained for protein, as described by Fairbanks *et al.* (31), except that the staining solution contained 0.25% Coomassie Blue.

Heme-associated peroxidase activity for detecting cytochrome P-450 was determined by the method of Thomas *et al.* (32). Prior to electrophoresis, the sample was dialyzed against 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 20% glycerol at 4°. The dialyzed sample was treated with 2% sodium dodecyl sulfate for 10 min at 0–4° and subjected to electrophoresis as described above. The electrophoresis was carried out in the dark. The gel was subsequently incubated and stained for peroxidase activity, as described previously (32). Under these experimental conditions, loss in stain intensity of cytochromes P-450$_I$ and P-450$_{III}$, but not of P-450$_{II}$, occurred within 72 hr. Therefore, the slab gel was scanned immediately after electrophoresis, using an LKB Zeineh soft laser scanning densitometer.

RESULTS

Changes in pulmonary microsomal catalytic activities and spectral properties induced by 3-MC. Previous studies have shown that pulmonary microsomes from untreated rabbits metabolize a variety of substrates (3, 6). In the present study, the effects of 3-MC on cytochrome P-450-dependent metabolic activities were determined using benzo[*a*]pyrene and ethylmorphine as substrates. As shown in Table 1, treatment with 3-MC caused a 2-fold induction of benzo[*a*]pyrene hydroxylase activity. In contrast, 3-MC pretreatment had no effect on ethylmor-

TABLE 1

Effect of 3-MC on catalytic activities and spectral properties of rabbit lung microsomal cytochrome P-450

3-MC, 25 mg/kg/day for 4 days, was administered i.p. to male New Zealand White rabbits. The animals were killed on day 5. Control animals received corn oil only. In each experiment, lungs from four rabbits in each treatment group were pooled, and washed microsomes were prepared. Each value represents the mean ± standard error of three experiments.

Assay	Controls	3-MC
Benzo[*a*]pyrene hydroxylase (nmoles OHBP/hr/mg protein)	0.82 ± 0.05	1.82 ± 0.10[a]
Ethylmorphine *N*-demethylase (nmoles HCHO/hr/mg protein)	37.1 ± 4.5	37.1 ± 5.9
Cytochrome P-450 content (nmoles/mg protein)	0.36 ± 0.05	0.42 ± 0.04
CO-difference spectrum absorbance maximum (nm)	451.1 ± 0.1	450.2 ± 0.2[a]
Ethyl isocyanide difference spectrum, 456:430 nm peaks)	0.67 ± 0.02	0.78 ± 0.04

[a] Value significantly different from respective control value ($p < 0.05$).

phine *N*-demethylase activity. The cytochrome P-450 content in pulmonary microsomes was 17% higher in 3-MC-treated rabbits when compared with the content of the hemeprotein in lungs of untreated rabbits; however, this difference was not statistically significant.

Previous studies (33) have shown that when ethyl isocyanide combines with reduced cytochrome P-450 the difference spectrum shows two Soret peaks at about 455 and 430 nm. Studies by Alvares *et al.* (25) have shown that, when compared with untreated rabbits, the CO-difference spectra of reduced liver microsomes from 3-MC-treated rabbits show a shift toward shorter wavelengths and an increase in the ratio of the 455:430 nm peaks of the ethyl isocyanide difference spectrum of liver microsomes. In the present studies, the spectral properties of cytochromes P-450 from lung microsomes obtained from untreated and 3-MC-treated rabbits were compared. As shown in Table 1, 3-MC caused a significant shift in the absorption maximum to a shorter wavelength in the CO-difference spectrum of lung microsomes. When ethyl isocyanide was used as a ligand with reduced microsomes, 3-MC did not cause a significant change in the ratio of the two peaks of the ethyl isocyanide-induced difference spectrum (Table 1).

Column chromatography of cytochromes P-450 isolated from lungs of untreated and 3-MC-treated rabbits. Microsomes were prepared from lungs of untreated and 3-MC-treated rabbits. The microsomal preparations, containing approximately equal amounts of protein, were solubilized using sodium cholate, dialyzed overnight, and chromatographed on DE-52 columns, as described under Materials and Methods. The column elution profiles of the hemeproteins from untreated and 3-MC-treated rabbits are shown in Fig. 1. The first peak, which was eluted with Emulgen 911, contained cytochrome P-450$_{I}$. A second peak, which was eluted at the beginning of KCl gradient, contained cytochrome P-450$_{II}$. The final peak contained NADPH-cytochrome *c* (P-450) reductase and, in data not shown, cytochrome b_5. In confirmation of previously published results of Wolf *et al.* (4), Fig. 1 (*top*) shows that, in untreated rabbits, two cytochromes, P-450$_{I}$ and P-450$_{II}$, were eluted from the column in approximately equal amounts. Fig. 1 (*bottom*) shows that, in 3-MC-treated rabbits, the first two peaks associated with cytochromes P-450 were qualitatively and quantitatively similar to the peaks obtained with untreated rabbits. In contrast, an additional peak of cytochrome P-450, termed P-450$_{III}$, was detected between the P-450$_{II}$ and reductase peaks. This peak was consistently obtained with all pulmonary microsomal fractions from 3-MC treated rabbits.

Table 2 shows the partial purification of cytochromes P-450 from 3-MC-treated rabbits. Lung microsomes were subjected to several purification steps. In the resolved forms of hemeproteins, the specific content of cytochrome P-450$_{III}$ was lower than those of cytochromes P-450$_{I}$ and P-450$_{II}$. The specific contents of cytochromes P-450$_{I}$ and P-450$_{II}$ were similar to the previously reported values obtained with untreated rabbits (13), indicating that 3-MC had a minimal effect, if any, on cytochromes P-450$_{I}$ and P-450$_{II}$.

Spectral properties of cytochromes P-450$_{I}$, P-450$_{II}$, and P-450$_{III}$. Figure 2 shows the CO-difference spectra of pulmonary cytochromes P-450 obtained from 3-MC-treated rabbits. The absorption maxima of cytochromes P-450$_{I}$ and P-450$_{II}$ occurred at 452 and 450 nm, respectively, similar to data previously reported (4, 13) for these

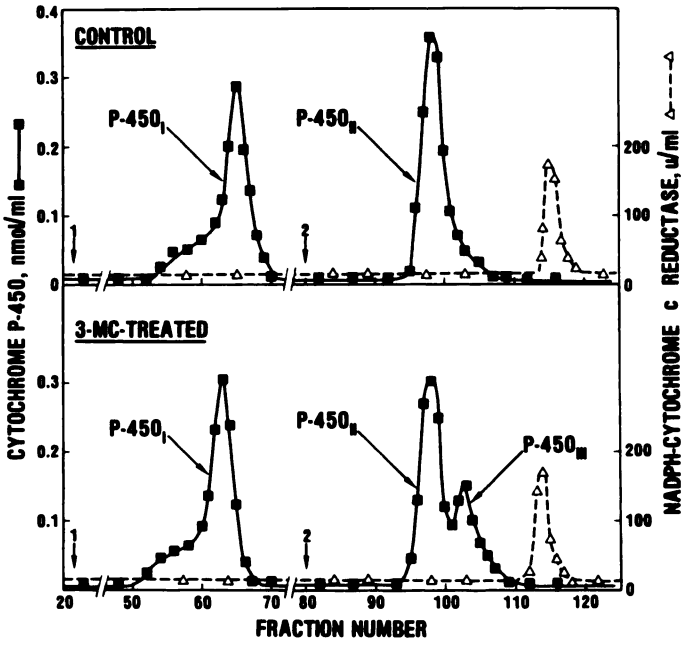

FIG. 1. *Column chromatography of solubilized lung microsomes from controls and 3-MC-pretreated rabbits*

Approximately 140 mg of microsomal protein were applied to Whatman DE-52 columns, previously equilibrated with 10 mM potassium phosphate buffer (pH 7.7) containing 20% glycerol (v/v), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1% sodium cholate. Both columns were run simultaneously. *Numbered arrows* represent changes in elution buffers: *1*, equilibration buffer containing 0.2% Emulgen 911; *2*, start of linear KCl gradient, 0–0.5 M KCl, in equilibration buffer containing 0.2% Emulgen 911. In data not shown, the profile of hemeprotein eluting from the column was monitored by determining the absorbance at 418 nm. Cytochrome P-450 and NADPH-cytochrome *c* reductase activities were determined as described under Materials and Methods.

TABLE 2

Partial purification of lung microsomal cytochromes P-450 from 3-MC-treated rabbits

Rabbits were treated and microsomes were prepared as described in legend to Table 1. Microsomal suspensions, equivalent to 30 g of lung (wet weight) were subjected to various purification steps, as described under Materials and Methods. Immediately after each purification step, the cytochrome P-450 content was determined in the sample. Each value represents the mean ± standard error of three experiments.

Step	Protein	Cytochrome P-450	
		Content	Specific content
	mg	*nmoles*	*nmoles/mg protein*
Microsomes	218.3 ± 8.1	73.8 ± 5.5	0.34 ± 0.01
Sodium cholate digestion	142.6 ± 11.4	52.2 ± 3.7	0.37 ± 0.01
DEAE-cellulose chromatography			
$P\text{-}450_I$	8.5 ± 1.0	18.3 ± 1.3	2.19 ± 0.13
$P\text{-}450_{II}$	13.2 ± 2.5	14.8 ± 1.4	1.18 ± 0.16
$P\text{-}450_{III}$	15.2 ± 3.6	4.8 ± 0.8	0.33 ± 0.04

cytochromes isolated from lung microsomes obtained from untreated rabbits. The absorption maximum of cytochrome $P\text{-}450_{III}$ occurred at about 448 nm. Figure 3 shows the ethyl isocyanide difference spectra of rabbit lung cytochromes P-450. The spectra of cytochrome $P\text{-}450_I$ from untreated and 3-MC-treated rabbits showed absorption maxima at 457 and 431 nm. For cytochrome $P\text{-}450_I$, the ratio of the peak heights at 457 and 431 nm was 0.81. In contrast, the spectra of cytochrome $P\text{-}450_{II}$ from untreated and treated rabbits showed absorbance peaks primarily at 455 nm, with minimal absorbance occurring at 431 nm. With cytochrome $P\text{-}450_{II}$, the ratio of the 455:431 nm peaks was 2.42. Figure 3 also shows the ethyl isocynaide difference spectrum of cytochrome

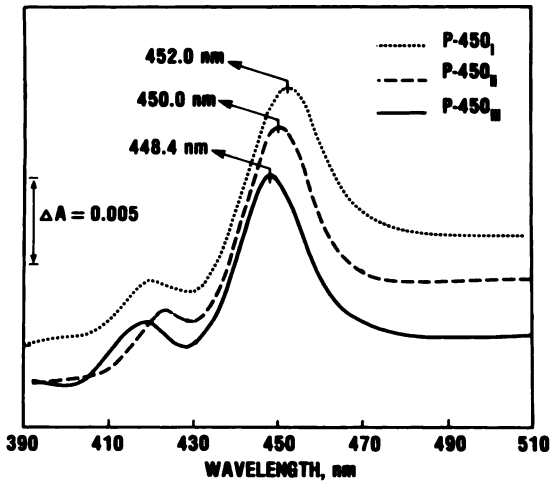

FIG. 2. *CO-difference spectra of cytochromes $P\text{-}450_I$, $P\text{-}450_{II}$, and $P\text{-}45_{III}$ obtained from lungs of 3-MC-treated rabbits*

The spectra were recorded after adding sodium dithionite to both the sample and reference cuvettes and then bubbling CO in the sample cuvette. For each determination, the cytochrome P-450 concentration was 0.1 nmole/ml of 10 mM phosphate buffer (pH 7.7) containing 20% glycerol (v/v), 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1% sodium cholate, and 0.2% Emulgen 911.

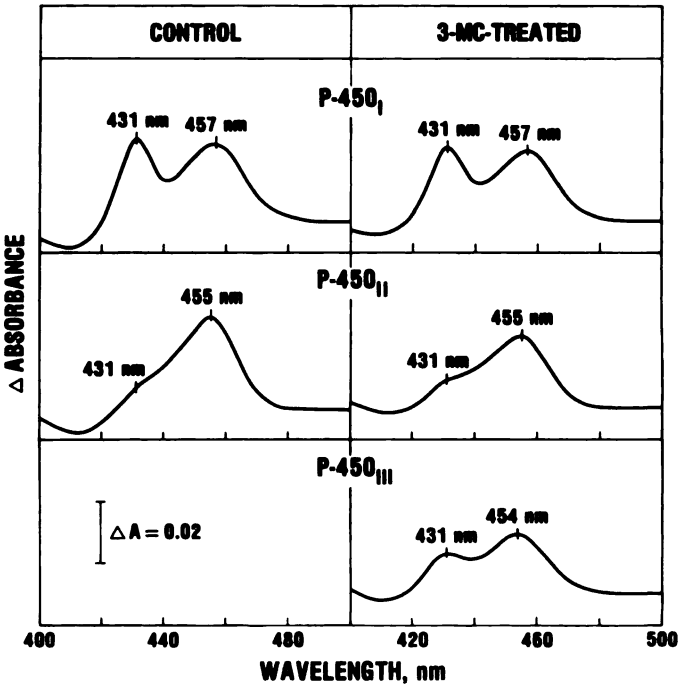

FIG. 3. *Ethyl isocyanide difference spectra of cytochromes P-450 isolated from lungs of controls and 3-MC-treated rabbits*

The spectra were recorded after reducing both the sample and reference cuvettes with sodium dithionite and then adding ethyl isocyanide (0.4 mM) to the sample cuvette. For each determination, the cytochrome P-450 concentration was 0.5 nmole/ml of 0.1 M phosphate buffer (pH 7.4).

$P\text{-}450_{III}$ from 3-MC-treated rabbits. The spectrum showed absorption maxima at 454 and 431 nm, and the ratio of the 454:431 nm peaks was 1.73. Therefore, the results of the ethyl isocyanide and CO-difference spectral determinations clearly show that cytochrome $P\text{-}450_{III}$ is different from $P\text{-}450_I$ and $P\text{-}450_{II}$.

Our previous studies (13) have shown that at room temperature the reduction rates and stabilities of cytochromes $P\text{-}450_I$ and $P\text{-}450_{II}$ isolated from lungs of rabbits pretreated with PCBs are different. In the present study, the *in vitro* stabilities of the three partially purified cytochromes isolated from lungs of 3-MC-treated rabbits were determined. In these experiments, the hemeproteins were reduced with dithionite, CO was bubbled into the sample cuvette, and CO-difference spectra were determined at various times depicted in Fig. 4. The CO-difference spectra of reduced cytochromes $P\text{-}450_I$ and $P\text{-}450_{III}$ attained absorbance maxima in less than 10 min, whereas about 20 min were required for cytochrome $P\text{-}450_{II}$ to reach maximal absorbance (Fig. 4). At 1 hr at room temperature, about 60% of $P\text{-}450_I$ and $P\text{-}450_{III}$ were degraded to their inactive form, cytochrome P-420. In contrast to the other two hemeproteins, cytochrome $P\text{-}450_{II}$ remained relatively stable (Fig. 4), only 15% being converted to cytochrome P-420 at 1 hr.

SDS-PAGE of partially purified cytochromes P-450 from rabbit lung. To confirm the inducibility of cytochrome $P\text{-}450_{III}$ by 3-MC in lung, the peaks associated with cytochromes P-450 eluted from DE-52 columns were concentrated and subjected to SDS-PAGE. As shown in *Wells B* and *C* of Fig. 5, the preparations of cytochromes $P\text{-}450_I$ from untreated and 3-MC-treated rabbits showed

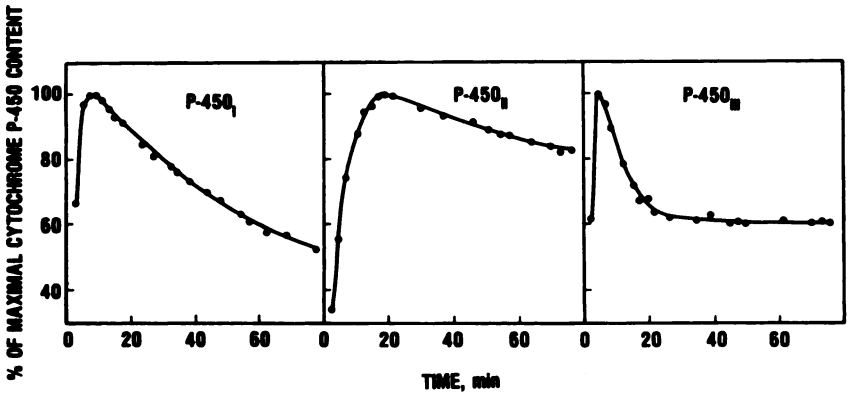

FIG. 4. *Difference in reduction rates and stabilities in cytochromes P-450$_I$, P-450$_{II}$, and P-450$_{III}$ from lungs of 3-MC-treated rabbits*
Sodium dithionite was added to reference and sample cuvettes at time 0. Carbon monoxide was bubbled into the sample cuvette at 1.8 min, and the CO-difference spectrum was determined at various time intervals for about 1 hr at room temperature.

two major protein-staining bands. The lower major band contained cytochrome P-450$_I$, since the band occurred at a molecular weight of 52,000, which agrees with the previously reported molecular weight for purified cytochrome P-450$_I$ (5–7). No significant difference was observed in the stainings of cytochromes P-450$_I$ from untreated and treated rabbits, indicating that 3-MC had no significant effect on cytochrome P-450$_I$. *Wells D* and *E* of Fig. 5 show the protein-staining patterns of partially purified preparations of pulmonary cytochrome P-450$_{II}$ from untreated and 3-MC-treated rabbits. Two major and several minor bands were observed in the region of

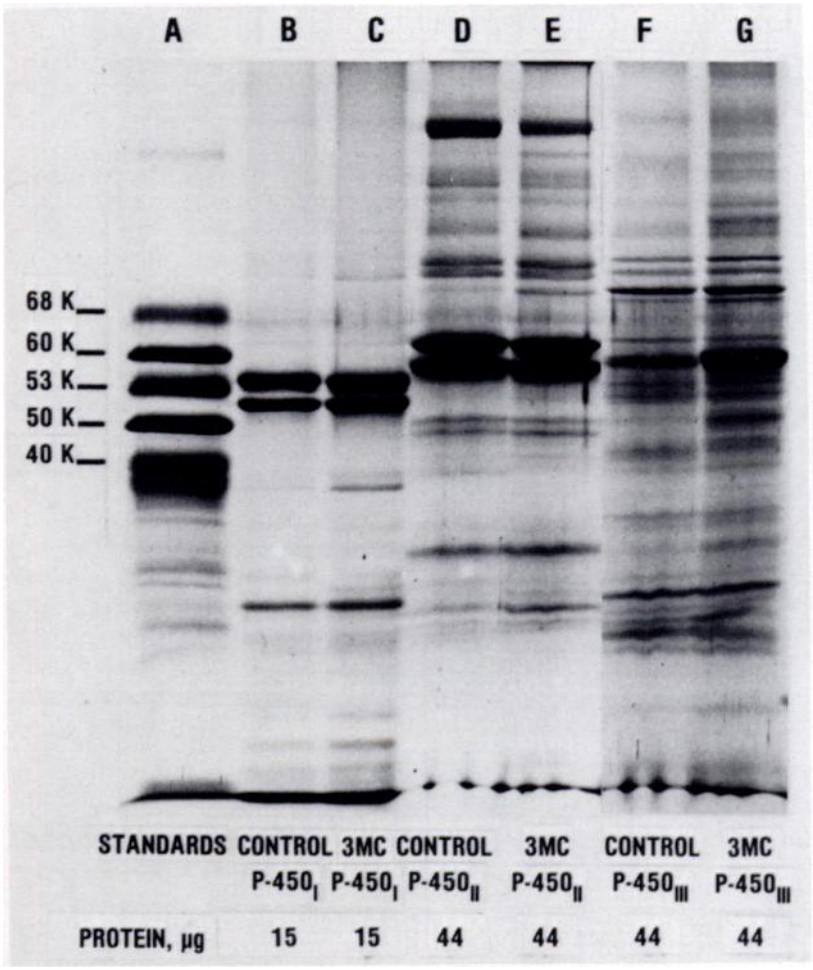

FIG. 5. *SDS-PAGE of cytochromes P-450 isolated from lungs of untreated and 3-MC-pretreated rabbits*
DE-52 column eluates corresponding to P-450$_I$, P-450$_{II}$, and P-450$_{III}$ peaks, respectively, were pooled, concentrated, and subjected to slab gel electrophoresis, as described under Materials and Methods. *Well A* contained protein standards having known molecular weights: bovine serum albumin, 68,000; catalase, 60,000; glutamate dehydrogenase, 53,000; fumarase, 50,000; and aldolase, 40,000. Values at the *bottom* represent micrograms of protein applied to the gels.

53,000–68,000 mol wt. The lower major band, at 57,000 mol wt, most likely contained cytochrome P-450$_{II}$ since this molecular weight agrees with the previously reported molecular weight value for this hemeprotein (5, 7). No significant differences were observed in the staining patterns for bands from untreated and 3-MC-treated rabbits, indicating that 3-MC had no significant effect on cytochrome P-450$_{II}$.

Although cytochrome P-450$_{III}$ was spectrally undetectable in DE-52 column eluates from untreated rabbits, for comparison purposes a control sample was prepared by pooling and concentrating the column fractions which eluted at the same position as cytochrome P-450$_{III}$. *Wells F* and *G* in Fig. 5 show the protein staining patterns of the control and cytochrome P-450$_{III}$ fractions from untreated and 3-MC-treated rabbits, respectively. The patterns were identical except that 3-MC pretreatment of rabbits caused a marked increase in protein staining at the 58,000 mol wt band (Fig. 5). These data strongly suggest that the marked increase in protein staining was associated with the induction of cytochrome P-450$_{III}$ by 3-MC.

Previous results (13) have shown that in untreated rabbits pulmonary cytochrome P-450$_{I}$ is less stable than P-450$_{II}$ in heme-associated peroxidase activity on SDS-PAGE gels. In the present study, a comparison of the peroxidase activities of the hemeproteins from 3-MC-treated rabbits was made. The densitometric results of the activity stainings are shown in Fig. 6. Cytochrome P-450$_{I}$, as well as cytochrome P-450$_{III}$, showed small peaks of peroxidase activities in the gel. With both of these hemeproteins, a large amount of free heme was detected at the bottom of the gel, below the tracking dye position. In contrast, cytochrome P-450$_{II}$ showed a large peak of peroxidase activity and a small amount of free heme. The data obtained with cytochromes P-450$_{I}$ and P-450$_{II}$ confirm previously published data (13). The present studies demonstrate that cytochrome P-450$_{III}$, like P-450$_{I}$, is less stable than P-450$_{II}$ in retaining heme-associated peroxidase activity.

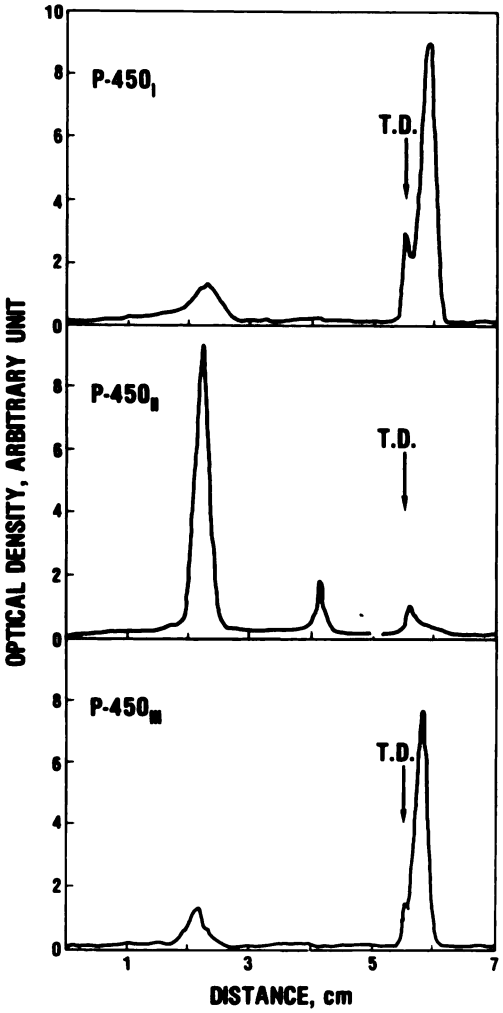

FIG. 6. *Densitometric determination of heme-associated peroxidase activity stainings of cytochromes P-450$_{I}$, P-450$_{II}$, andP-450$_{III}$ isolated from lungs of 3-MC-pretreated rabbits*

Cytochromes P-450 preparations were dialyzed overnight to remove dithiothreitol, subjected to SDS-PAGE, and peroxidase activity was determined by the method of Thomas *et al.* (32). Twenty picomoles of hemeprotein were applied to each gel. T.D., Tracking dye.

DISCUSSION

Previous studies have shown that PCBs are "mixed" types of inducers, and that the spectral and catalytic properties of cytochromes P-450 induced in rat and rabbit livers by PCBs are similar to those elicited when experimental animals are treated with the phenobarbital and 3-MC classes of inducing substances (23, 34–38). However, recent studies (12, 13) have shown that, in contrast to the effects on rat lung, PCB pretreatment resulted in inhibition of benzo[*a*]pyrene hydroxylase and ethylmorphine *N*-demethylase activities in rabbit lungs. Moreover, the PCBs caused a selective decrease in pulmonary cytochrome P-450$_{I}$ in rabbit lungs (13). The present studies were carried out to determine whether such a selective loss of pulmonary cytochrome P-450 occurred in lungs of rabbits pretreated with 3-MC.

The data obtained in the present studies with 3-MC-treated rabbits differ from those obtained with rabbits pretreated with the PCB mixture, Aroclor 1254. In contrast to rabbits pretreated with PCBs, 3-MC treatment did not result in a decrease in total pulmonary cytochrome P-450. The present studies demonstrate that 3-MC induces a new form of cytochrome P-450, termed cytochrome P-450$_{III}$, to distinguish the latter from cytochromes P-450$_{I}$ and P-450$_{II}$, previously isolated from lungs of untreated rabbits (3–6) and PCB-treated (13) rabbits. In confirmation of previous studies (12), 3-MC treatment caused a significant increase in benzo[*a*]pyrene hydroxylase activity and a minimal increase in the cytochrome P-450 content of rabbit lung (Table 1). A consistent shift toward shorter wavelengths was observed in the absorbance maximum of the CO-difference spectrum of pulmonary microsomes obtained from the 3-MC-treated rabbits. In previously reported data, Litterst *et al.* (19) observed a significant increase in cytochrome P-450 content, but Smith *et al.* (20) did not observe any increases in cytochrome P-450 content and benzo[*a*]pyrene hydroxylase activity in lungs of 3-MC treated rabbits. These discrepancies may be due to differences in age and 3-MC treatment regimen of rabbits, among other factors.

The spectral changes in the CO- and ethyl isocyanide-

induced difference spectra of the lung microsomes were of a smaller magnitude that those observed with livers from 3-MC-treated rabbits. These observations and the marginal increase in microsomal cytochrome P-450 indicated that 3-MC either induced a minor form of cytochrome P-450, or it induced a major form of the hemeprotein at the expense of another cytochrome P-450. The latter possibility arose from previously published results by Liem *et al.* (11), who showed that induction of a new form of pulmonary cytochrome P-450 by TCDD is accompanied by a depression of another microsomal protein. The results of partial purification of pulmonary cytochrome P-450 (Fig. 1, Table 2) demonstrate that 3-MC induces a new form of cytochrome P-450 in lung, without significantly affecting the contents of cytochromes $P\text{-}450_I$ and $P\text{-}450_{II}$.

Cytochrome $P\text{-}450_{III}$ is different from cytochromes $P\text{-}450_I$ and $P\text{-}450_{II}$ with respect to spectral characteristics as well as SDS-PAGE gel patterns. In the CO-difference spectral determinations, cytochromes $P\text{-}450_I$, $P\text{-}450_{II}$, and $P\text{-}450_{III}$ showed absorption maxima about 452, 450, and 448 nm, respectively (Fig. 2). In the ethyl isocyanide difference spectral determinations, the 455 nm region peaks of cytochromes $P\text{-}450_I$, $P\text{-}450_{II}$, and $P\text{-}450_{III}$ showed absorption maxima at 457, 455, and 454 nm, respectively (Fig. 3). The ratio of 454:431 nm peaks of cytochrome $P\text{-}450_{III}$ was between those of $P\text{-}450_I$ and $P\text{-}450_{II}$ (Fig. 3). In addition, cytochromes $P\text{-}450_I$ and $P\text{-}450_{III}$ had faster reduction rates and were less stable than $P\text{-}450_{II}$ (Fig. 4). SDS-PAGE of the cytochromes showed that the molecular weights of cytochromes $P\text{-}450_I$, $P\text{-}450_{II}$, and $P\text{-}450_{III}$ were about 52,000, 57,000 and 58,000, respectively (Fig. 5). Also, the heme-associated peroxidase activity studies showed that cytochromes $P\text{-}450_I$ and $P\text{-}450_{III}$ retained less heme than did $P\text{-}450_{II}$ following electrophoresis (Fig. 6). These studies demonstrate that the cytochromes are spectrally and electrophoretically distinct forms of hemeproteins. By the same criteria, the studies also show that 3-MC has no significant effect on cytochromes $P\text{-}450_I$ and $P\text{-}450_{II}$.

In rabbits, lung cytochrome $P\text{-}450_I$ has been shown to be identical with liver cytochrome P-450 Form 2 ($P\text{-}450_{LM_2}$) with respect to several parameters (3, 6, 7, 10). Lung cytochrome $P\text{-}450_{II}$ appears to be immunochemically distinct from liver Form 4 ($P\text{-}450_{LM_4}$), the major liver microsomal form of cytochrome P-450 induced in adult rabbits by 3-MC (6). TCDD-inducible lung cytochrome P-450 appears to be identical with rabbit liver Form 6 ($P\text{-}450_{LM_6}$), using several criteria (11). It is of interest to note that rabbit liver Form 6 is several-fold more active than Forms 2 and 4 in metabolizing polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene (39). Thus, it will be important to determine whether the 3-MC-inducible cytochrome $P\text{-}450_{III}$ is identical with or similar to Form 6 of rabbit liver cytochrome P-450. In summary, the results of the chromatographic separation and spectral and electrophoretic studies described in this report demonstrate that 3-MC has the ability to induce a new and distinct form of cytochrome P-450 in rabbit lung. The induced hemeprotein needs to be further characterized with respect to its immunochemical and substrate specificities, and possible differences in cell type localization in the lung.

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