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# SPECIFICITY OF A PHENOBARBITAL-INDUCED CYTOCHROME P-450 FOR METABOLISM OF CARBON TETRACHLORIDE TO THE TRICHLOROMETHYL RADICAL*

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**Abstract**—Evidence is presented which demonstrates that the first polypeptide to disappear in liver microsomes of phenobarbital-induced rats treated with $CCl_4$ was the 52,000 dalton P-450 cytochrome. Data are also presented which show that this form of cytochrome P-450 was capable of generating the trichloromethyl radical from $CCl_4$ in a reconstituted system containing the purified cytochrome, NADPH–cytochrome P-450 reductase, NADPH, $CCl_4$, and the spin-trapping agent, phenyl-t-butyl nitrone. Other cytochrome P-450 fractions not containing the 52,000 dalton form did not produce this radical. The formation of this highly reactive radical may have resulted in localized damage to the cytochrome, causing the cytochrome either to be released from the microsomal membrane or to form large aggregates which did not migrate in the gel electrophoretic procedures employed.

The studies in this report were done to obtain further information on the relationship between the metabolism of carbon tetrachloride ($CCl_4$) and the early loss of specific forms of cytochrome P-450 which has been described in the accompanying report [1]. The relationship between the specific loss of cytochromes and the capacity of the different cytochrome P-450 fractions to metabolize $CCl_4$ to trichloromethyl ($\cdot CCl_3$) radicals in reconstituted systems was examined. The electron spin resonance (e.s.r.) studies involved a technique developed in this laboratory which makes use of a spin-trapping agent that permits the unequivocal identification of the $\cdot CCl_3$ radical [2]. Although all of the cytochrome P-450 fractions had hydroxylating activity in reconstituted systems, only the purified 52,000 dalton cytochrome P-450 or fractions which contained the 52,000 polypeptide resulted in the production of the $\cdot CCl_3$ radical in reconstituted monooxygenase systems which was detectable by e.s.r. The radical which was produced by this particular cytochrome was identical to that formed during the metabolism of $CCl_4$ by microsomes *in vitro* and by the liver *in vivo* [3]. This is the same cytochrome which was observed to essentially disappear in liver microsomes of rats within 30–40 min after treatment with $CCl_4$ *in vivo* [1]. Since this particular cytochrome is induced by prior treatment of rats with phenobarbital [4], the observed effects are probably associated with the increased toxicity of rats to $CCl_4$, which results from the introduction with phenobarbital [5, 6]. A possible role of lipid peroxidation in the events leading to the specific early loss of hemoprotein is proposed.

## MATERIALS AND METHODS

### *Materials*

Diethylaminoethyl cellulose (DE-52) was purchased from the Whatman Chemical Co., Maidstone, U.K.; phenobarbital sodium was purchased from Merck, Sharp & Dohme, Rahway, NJ; aniline, sodium cholate, sodium deoxycholate, phenol reagent solution, ammonium sulfate and potassium chloride were obtained from the Fisher Chemical Co., Dallas, TX; phenyl-t-butyl nitrone (PBN) was obtained from Eastman Organic Chemicals, Rochester, NY; NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, cytochrome *c*, and glycerol were obtained from the Sigma Chemical Co., St. Louis, MO. 3,2,6,6-Tetramethyl piperidinoxy was a product of the Aldrich Chemical Co., Milwaukee, WI. Amberlite XAD-2 beads were purchased from Mallinckrodt; St. Louis, MO; ammonium persulfate, *N,N'*-methylene-bis-acrylamide, acrylamide, bromophenol blue, sodium dodecysulfate and hydroxyapatite were products of Bio-Rad Laboratories, Richmond, CA. Emulgen 911 was purchased from the Kao-Atlas Co., Ltd., Tokyo, Japan. Agarose-hexane-adenosine 2',5'-diphosphate (AG 2'5'-ADP type 2) from P-L Biochemicals, Milwaukee, WI; polyethylene glycol 6000 from the J. T. Baker Co., Phillipsburg, NJ; $CCl_4$ from the Eastman Kodak Co., Rochester, NY; and $^{13}CCl_4$ (90 atom % $^{13}C$) from Stohler Isotope Chemicals and Merck, Sharp & Dohme, Waltham, MA.

### *Methods*

*Preparation of cytochrome P-450.* Male, Sprague–Dawley rats (250–300 g body weight)

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were used in all experiments. Phenobarbital-inducible forms of cytochrome P-450 were purified from the liver microsomes of phenobarbital-pretreated male rats according to the method of West *et al.* [4], with minor modifications. Rats were given phenobarbital intraperitoneally at a dose of 70 mg/kg of body weight/day for 4 days. Microsomes were prepared in 1.15% KCl and the pellet from the final centrifugation was resuspended in 0.25 M sucrose so that the microsomes from 1 g of liver were dispersed in 0.5 ml of the sucrose solution. The protein concentration of the microsomal suspensions and fractions thereof was determined by the method of Lowry *et al.* [7]. The suspensions were solubilized with sodium cholate and fractionated according to the method of West *et al.* [4], except that 8% and 16% polyethylene glycol was used. The 16% polyethylene glycol mixture was stirred for 10 min at 4° and then centrifuged for 60 min at 105,000 *g*. The supernatant fraction was retained for preparation of NADPH–cytochrome P-450 reductase. The pellet was dissolved in 70–100 ml of buffer A (10 mM phosphate buffer*, pH 7.4, containing 0.1 mM EDTA, 20% glycerol, 0.5% sodium cholate and 0.2% Emulgen). The preparation was applied to a Whatman DE-52 column (2.5 × 50 cm) which had been equilibrated with buffer A. The column was then eluted overnight with 500–800 ml of buffer A containing 25 mM KCl, monitoring all fractions for absorbance at 416 nm. Fractions A and B were collected from this procedure. Remaining on the column was an intensely red band, 3–4 cm in width, which had migrated down approximately half way. The column packing was extruded and the red band cut out. The latter was suspended in buffer A and reapplied to a second DE-52 column (2.5 × 10 cm) which had been equilibrated with buffer A. This column was eluted with a linear gradient consisting of 250 ml of buffer A containing 25 mM KCl and 250 ml of buffer A containing 100 mM KCl. Fractions eluted having an absorbance at 416 nm of greater than 0.6 were combined and designated as Fraction C. Fraction C preparations were dialyzed overnight against 10 mM phosphate buffer, pH 7.4, containing 20% glycerol, 0.2% Emulgen and 0.1 mM EDTA before being applied to the hydroxyapatite column. The 52,000 dalton polypeptide (Fraction $C_1$) was separated by elution from the column with 100–150 ml of 50 mM phosphate buffer, of 75 mM phosphate buffer, and then 100–150 ml of 400 mM phosphate buffer, pH 7.4, containing 20% glycerol, 0.2% Emulgen and 0.1 mM EDTA. This polypeptide appeared as a single band on gel electrophoresis. This component, as well as Fraction C, had a reduced CO absorbance spectrum maximum at 450 nm, was positive for heme staining when subjected to electrophoresis without prior heating with mercaptoethanol, and had monooxygenase activity in a reconstituted system described below. To remove the detergent from the cytochrome P-450 fractions in preparation for storage, the fractions were applied to hydroxyapatite columns eluted with 0.4 M phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. The eluted fractions were treated in batch with Amberlite and then dialyzed overnight against 50 mM phosphate buffer, containing 0.1 mM EDTA, 20% glycerol and 0.1 mM dithiothreitol at 4°. The concentrations of various cytochrome P-450 fractions were determined by the method of Omura and Sato [8] from the reduced, CO-complexed difference spectrum, using an extinction coefficient of 91 $mM^{-1}$ $cm^{-1}$. The specific content of cytochrome P-450 in the various fractions was expressed as nmoles per mg of protein. Cytochrome P-450 ranged from 5 to 8 for cytochrome P-450 fractions A and B, and from 12 to 16 for cytochrome P-450 Fractions C, $C_1$ and $C_2$. The monooxygenase activity of each hemoprotein fraction was assayed in reconstituted systems by measuring aniline hydroxylation [9]. The reaction mixture (1.0 ml final volume) contained the following components: 3.00 nmoles of purified P-450 fraction; 600 units of purified NADPH–cytochrome P-450 reductase; 0.3 mM NADPH or an NADPH-generating system (5 μmoles glucose-6-phosphate, 0.3 μmole NADP, and 0.5 Kornberg units of glucose-6-phosphate dehydrogenase per ml reconstituted system); 50 μg dilauroyl glyceryl-3-phosphorylcholine, and 5 μmoles aniline, all in 0.05 M Tris–HCl buffer, pH 7.5. The incubation was carried out for 45 min at 37°. In reference systems in which fresh liver microsomes were employed instead of the reconstituted reductase and hemoprotein fractions, 3.0 mg of microsomal protein was used.

*NADPH–cytochrome P-450 reductase.* This enzyme was prepared by the method of French and Coon [10] with minor modifications. The supernatant fraction retained from the 16% polyethylene glycol treatment of solubilized microsomes described above was used for the reductase purification. The precipitate from the final fractionation was dissolved and dialyzed against 0.5 mM Tris–HCl, pH 7.7, 10% glycerol and 0.1 mM EDTA. Sodium deoxycholate was added to a final concentration of 0.1%, and the mixture was applied to a Whatman DE-52 column (2.5 × 40 cm) as described by French and Coon [10]. In the final step, fractions having reductase activity were combined and applied to a hydroxyapatite column and eluted with 0.4 M phosphate buffer, pH 7.7. The eluate was dialyzed overnight against 50 mM Tris–HCl buffer, pH 7.7, containing 10% glycerol, 0.1 mM EDTA and 0.1 mM dithiothreitol. This preparation, which showed a single band on gel electrophoresis, was employed in the reconstitution studies.

*Gel electrophoresis.* Polyacrylamide slab gel electrophoresis was carried out at 10° in the presence of sodium dodecylsulfate using an apparatus from Hoefer Scientific Instruments (model SE-500), with a discontinuous buffer system according to the method of Laemmli [11]. Microsomal samples were prepared at a final protein concentration of 0.5 mg/ml. Purified cytochrome P-450 fractions and NADPH–cytochrome P-450 reductase were prepared for electrophoresis at a protein concentration of 0.1 to 0.2 mg/ml in 0.06 M Tris–HCl buffer, pH 6.8, containing 1% sodium dodecylsulfate, 1.2% mercaptoethanol, and 12% glycerol. Bromophenol blue (0.001%) was used as the tracking dye in all samples.

* The buffer was composed of 3 parts 0.15 M $K_2HPO_4$ and 1 part 0.15 M $KH_2PO_4$.

Before pipetting the prepared samples into the gel slabs, they were placed in a boiling water bath for 3 min. The preparation and fixation of the slab gels were performed according to the procedure of Haugen and Coon [12]. Proteins of known molecular weight were used as electrophoretic markers. These included bovine serum albumin (68,000 mol. wt), catalase (58,000 mol. wt), glutamate dehydrogenase (53,000 mol. wt), and ovalbumin (45,000 mol. wt).

*Procedure for Ouchterlony double immunodiffusion studies.* Antiserum to the purified 52,000 dalton cytochrome P-450 was obtained following immunization of young male White New Zealand rabbits. Rats were treated with phenobarbital (70 mg/kg, i.p.) for 4 days. Liver microsomes were prepared, and the 52,000 dalton cytochrome P-450 (Fraction $C_1$) was purified to homogeneity as described under Methods. The purified cytochrome appeared as a single band when subjected to polyacrylamide slab gel electrophoresis. This preparation was used to prepare an antiserum. A solution of the purified cytochrome (0.5 mg protein in 0.5 ml of 0.01 M ammonium carbonate, pH 8.0) was added to 0.5 ml of complete Freund's Adjuvant (Difco). One injection of 0.5 ml was given intraperitoneally and 0.5 ml was injected intradermally into four sites on the back. Three weeks later, a booster of the purified cytochrome (0.1 mg protein in 0.25 ml ammonium carbonate mixed 1:1 with complete Freund's Adjuvant) was given intraperitoneally. The animals were bled by cardiac puncture 1 week after the last antigen injection under light Ketamine HCl anesthesia (35 mg/kg). Serum was collected and stored in 1-ml aliquots at +70° until used for assays. The presence of anti-52,000 dalton cytochrome P-450 antibody was demonstrated by a modification of the immunodiffusion technique of Ouchterlony [13]. The modification consisted of using micro-Ouchterlony plates (Sebia, Paris).

After desalting the gels, they were then dried under a flow of warm air. Visualization of precipitin lines in the gels was done with Crowles' Immunodifusion Double Stain (Polysciences, Warrington, PA) for 1 hr followed by destaining in absolute methanol–glacial acetic acid (9:1).

Clearly defined precipitin lines appeared using concentrations of the 52,000 dalton cytochrome P-450 as low as 0.05 nmole (the smallest amount tested).

*Enzymatic assays.* The reductase was assayed routinely by its ability to catalyze cytochrome *c* reduction in 0.3 M phosphate buffer, pH 7.7, at 25° [14]. The reaction was initiated by the addition of the reductase and the rate of cytochrome *c* reduction was determined at 550 nm, using an extinction coefficient of 21 $mM^{-1}$ $cm^{-1}$ for the conversion of the oxidized cytochrome to the reduced form [15]. One unit of enzyme is defined as the amount which catalyzes the reduction of 1 nmole of the cytochrome *c*/min under the conditions referred to above. The specific activity of the preparation is defined as units/mg protein. The activity of enzyme used in these studies was 31,500 units/mg protein.

*Electron spin resonance studies.* The reconstituted enzyme systems which were employed in the e.s.r. studies for detecting the generation of the $\cdot CCl_3$ radical were composed of 3.0 nmoles of the particular purified cytochrome P-450 fraction to be assayed, 0.14 M phenyl-t-butyl nitrone (PBN), 600 units of purified cytochrome *c* reductase, 0.3 mM NADPH or NADPH-generating system (described above), and 20 μl (0.2 mM) $CCl_4$ or $[^{13}C]CCl_4$ in 0.05 M phosphate buffer, pH 7.4. The final volume of all systems was 1.0 ml. The systems were incubated at 37° for 15 min. The mixture was then vortexed, placed in a Pasteur pipette with a sealed tip, and centrifuged at 500 *g* for 3 min. The e.s.r. spectra of the systems were determined with a Varian E-9 spectrometer equipped with an X-band (E-101-15) microwave bridge. The instrument settings were: microwave power, 25 MW; modulation amplitude, 1 G; time constant, 10 sec; scan range, 100 G. Spectral measurements were made at 24°.

## RESULTS

*Fractionation of phenobarbital-induced rat liver microsomal cytochrome P-450.* Slab gel electrophoresis of Fractions A, B, and C resulted in the banding patterns shown in Fig. 1. These fractions all had a reduced CO complex absorbance maximum at 450 nm (lanes D, E, and F respectively). The phenobarbital-induced 52,000 and 54,000 dalton components were apparently present only in Fraction C. Fractions A and B each showed several heme-staining bands after electrophoresis without the reduction and heating steps [16]. Fraction C showed only two heme-staining bands in positions not observed in Fractions A and B. Fraction C was subjected to an additional chromatographic procedure (see Methods) which resulted in purification of the 52,000 dalton component as an apparently homogenous protein, Fraction $C_1$ (Fig. 2). The $C_1$ fraction was positive on staining for heme and had a reduced Co complex absorbance at 450 nm. Another fraction ($C_2$) was recovered during the preparation of Fraction $C_1$ which appeared to be primarily the 54,000 dalton cytochrome, but it still contained an observable band for the 52,000 dalton component.

Fractions A, B, C and $C_1$ were assayed in reconstituted systems for their monooxygenase activity with aniline as substrate. Table 1 shows that all cytochrome P-450 fractions in the reconstituted systems were active in the production of *p*-hydroxyaniline, including the purified 52,000 dalton cytochrome P-450 (Fraction $C_1$). Omission of any component of the reconstituted system resulted in no activity.

*Ouchterlony immunodiffusion studies on the cytochrome P-450 fractions.* Rabbit anti-Fraction $C_1$ antiserum was prepared as described under Methods. When Ouchterlony immunodiffusion studies were performed with the various fractions and the antibody preparation, only Fraction C and its subfractions, $C_1$ and $C_2$, exhibited immunoprecipitin lines with the antiserum (Fig. 3). Neither Fractions A nor B showed precipitin lines, indicating that there was very little, if any, of the 52,000 dalton cytochrome P-450 in these two fractions (Fig. 3).

The $C_2$ fraction produced an immunoprecipitin band with the antiserum to the 52,000 dalton cytochrome which had a line of identity with the precip-

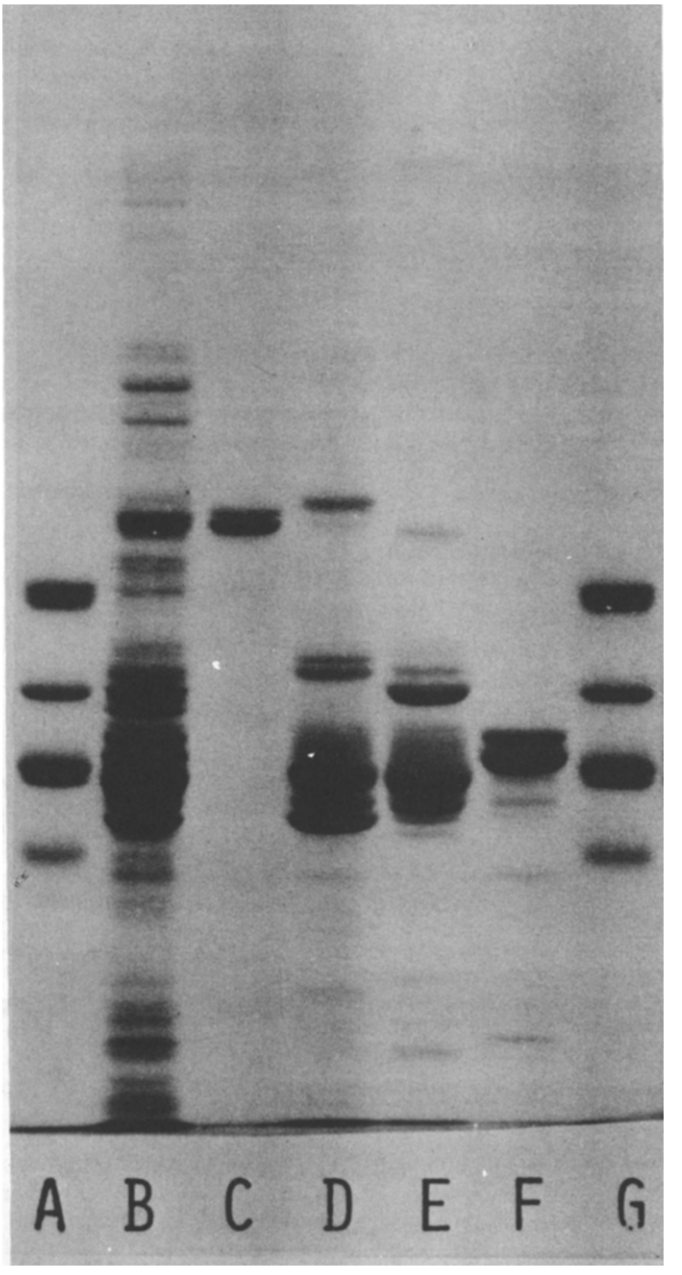


Fig. 1. Sodium dodecylsulfate polyacrylamide slab gel electrophoresis of three different phenobarbital-induced rat liver microsomal cytochrome P-450 fractions and purified NADPH–cytochrome P-450 reductase. Well A: 0.5 μg of each protein standard with molecular weights of 68,000 (bovine serum albumin), 58,000 (catalase), 53,000 (glutamate dehydrogenase), and 45,000 (ovalbumin) from top to bottom. Well B: 10 μg of microsomal protein from a phenobarbital-treated rat. Well C: 0.5 μg of purified phenobarbital liver microsomal NADPH–cytochrome P-450 reductase. Well D: 2 μg of Fraction A. Well E: 2 μg of Fraction B. Well F: 1 μg of Fraction C. Well G: protein standards (as in Well A). Conditions of the electrophoretic separation are described under Methods.

itin band of the latter cytochrome (Fig. 3). This indicated that this fraction, which consisted primarily of the 54,000 dalton cytochrome fraction, also contained some of the 52,000 dalton form, in agreement with the electrophoretic analysis. Further purification of the $C_2$ fraction was not done. A purified fraction of a 56,000 dalton cytochrome P-450 obtained from liver microsomes of Aroclor 1254-treated animals produced no immunoprecipitin line with the anti-fraction $C_1$ serum (Fig. 3).

*Trichloromethyl radical production in reconstituted systems.* In all of the reconstituted enzyme systems, the same quantity of purified cytochrome P-450 (based on the absorbance of the reduced CO complexes) and the same amount of reductase activity were added to each system. Incubations of rat liver

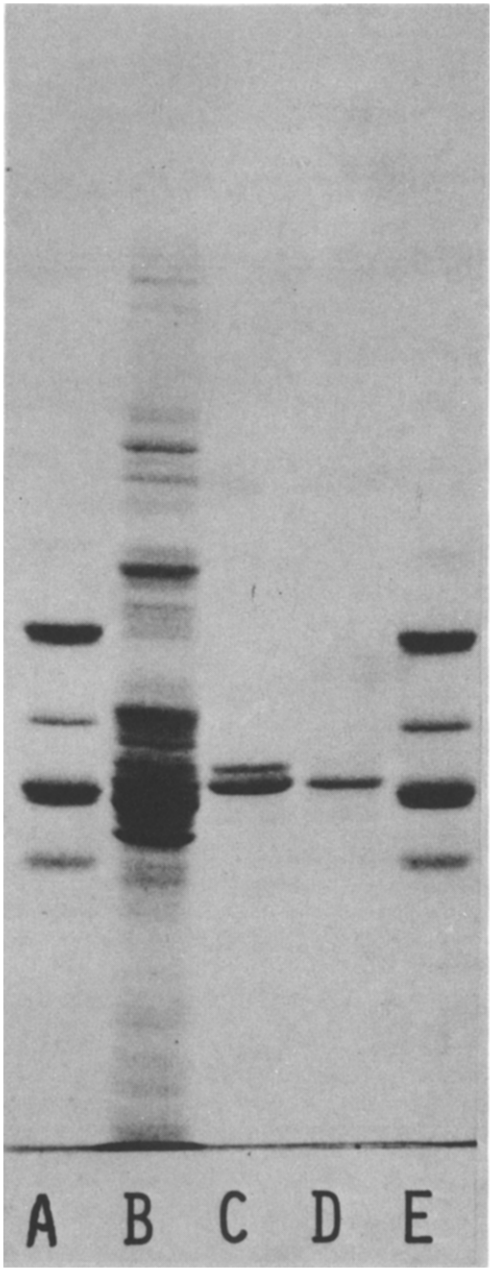


Fig. 2. Sodium dodecylsulphate polyacrylamide slab gel electrophoresis of purified phenobarbital-induced rat liver microsomal cytochrome P-450 Fraction $C_1$ (52,000 daltons). Well A: protein standards as described in the legend of Fig. 1. Well B: 10 μg of solubilized phenobarbital-induced rat liver microsomal protein. Well C: 1 μg of Fraction C. Well D: 1 μg of Fraction $C_1$. Well E: same as Well A.

microsomes with $CCl_4$, the spin-trap (phenyl-t-butyl nitrone), and an NADPH-generating system were performed as a reference reaction for the reconstituted systems. There was a rapid generation of the $\cdot CCl_3$ radical adduct signal in microsomal systems (Fig. 4A). When either Fraction A or B was assayed in a reconstituted system containing $CCl_4$ and the spin-trapping agent (PBN), no detectable e.s.r. signal was obtained (Fig. 4H and 4I). However, when the system was reconstituted using Fraction C as the source of cytochrome P-450, a strong signal due to the formation of the $\cdot CCl_3$ radical was obtained (Fig. 4B). This fraction contained the 52,000 and 54,000 dalton P-450 cytochromes while Fractions A and B did not contain immunologically or electrophoretically detectable amounts of these cytochrome components. Formation of the $\cdot CCl_3$ radical was shown to be dependent on the enzymatic functioning of the reconstituted mixed function oxidase system by the following studies. Heating Fraction C at 70° for 5 min prior to addition to the reconstituted systems eliminated the metabolism of $CCl_4$ to the $\cdot CCl_3$ radical (Fig. 4E). Omitting either the reductase or Fraction C from the reaction system also resulted in no signal production (Fig. 4C and 4D respectively). The reaction was also inhibited by a sulfhydryl reagent, *p*-chloromercuribenzoate (Fig. 4F). In addition, inhibitors of mixed function oxidase activity, such as carbon monoxide (Fig. 4G), SKF-25A, and sodium dodecylsulfate, also abolished the signal.

*Magnitude of radical trapping.* Calculation of the amount of $\cdot CCl_3$ radical which was spin-trapped by PBN was performed using a stable nitroxyl radical, 3,2,6,6-tetramethyl piperidinoxy, as a standard for estimating the nitroxyl radical functions in the $\cdot CCl_3$ radical adduct [17]. This procedure involves quantification of the amount of free radicals by integration of the derivative spectra using the Reiman sum procedure [18]. The results of the integration indicate that in the reconstituted systems employing Fraction C, for example, approximately 0.6% of the total amount of $CCl_4$ added to the reaction system appeared to be converted to the radical. This is equivalent to an average of 1.25 nmoles of $\cdot CCl_3$ per ml of reaction system if one assumes a uniform distribution of the radicals at the time of assay. Since it may be presumed that the efficiency of radical trapping by PBN was less than 100%, the total quantity of radicals produced in this system may be somewhat greater.

*Parameters influencing radical production.* Radical production was a function, within limits, of the concentration of $CCl_4$ (Fig. 5) and the amount of Fraction C cytochrome P-450 (Fig. 5) added to the reaction system. The signal also increased in intensity as the concentration of the reductase was increased up to about 600 units (Fig. 5).

Fractions $C_1$ and $C_2$, obtained from further

Table 1. Aniline hydroxylation by fractions of partially purified cytochrome P-450 liver microsomes of phenobarbital-induced rats*

| Source of cytochrome P-450 | Aminophenol formation (nmoles/nmole cytochrome P-450) |
|---|---|
| Liver microsomes† | 5.33 |
| Fraction A | 5.11 |
| Fraction B | 1.75 |
| Fraction C | 8.12 |
| Fraction $C_1$ | 4.93 |

* The compositions of the reconstituted systems testing each of the fractions and the conditions of the incubations are described under Methods.

† Pooled rat liver microsomes from which the fractions were prepared.

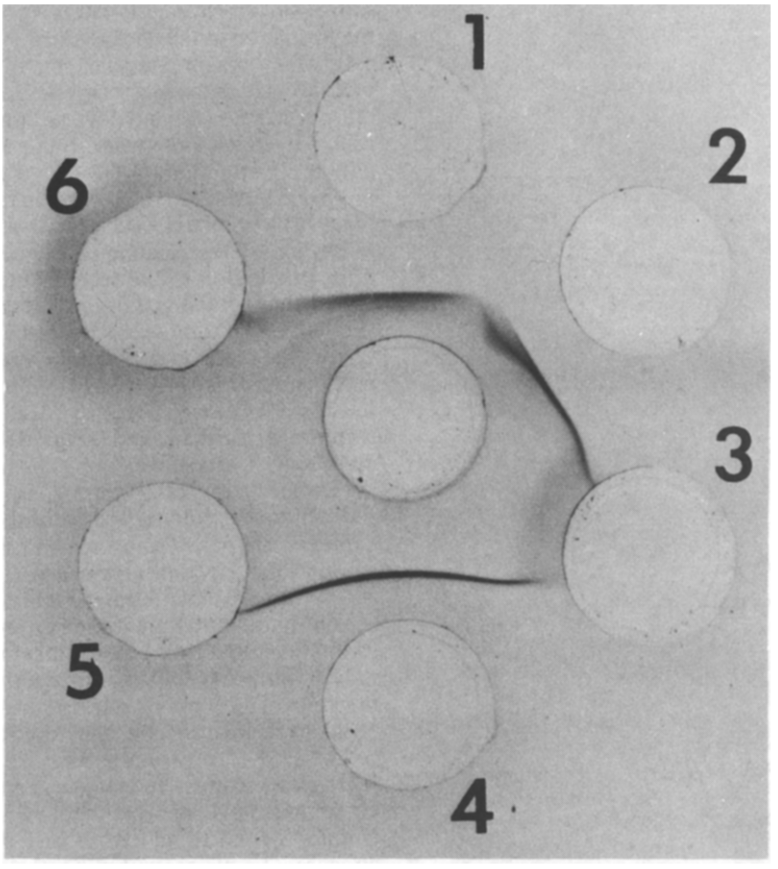


Fig. 3. Ouchterlony immunodiffusion analysis of purified cytochrome P-450 fractions from phenobarbital-induced rat liver microsomes. The cytochrome P-450 content in each well is indicated in nmoles. Well 1: Fraction $C_1$, 0.05 nmole. Well 2: Fraction $C_2$, 0.1 nmole. Well 3: purified cytochrome P-450 (56,000 daltons) from Aroclor 1254-induced rat liver microsomes, 0.1 nmole. Well 4: Fraction $C_1$ from a different preparation, 0.1 nmole. Well 5: Fraction A, 0.1 nmole. Well 6: Fraction B, 0.1 nmole. The procedure employed in this analysis is described under Methods.

chromatography of Fraction C, were tested for their capacities to metabolize $CCl_4$ in reconstituted systems containing PBN. Fraction $C_1$ (the 52,000 dalton cytochrome P-450) produced a substantial yield of the $\cdot CCl_3$ radical adduct (Fig. 6C). Fraction $C_2$ also produced the radical, but not at the high level catalyzed by Fraction $C_1$, based on the same cytochrome P-450 content (Fig. 6D).

To ascertain whether or not the lack of activity of Fractions A and B in producing the $\cdot CCl_3$ radical was due to an inadequate amount of phospholipid in the purified fractions, signal generation was tested after addition of phosphatidyl choline to reconstituted systems containing either Fraction A or B. The results remained negative. Increasing the amounts of Fraction A or B in the system also resulted in no signal. Since these two fractions had been shown to have mixed function oxidase activity in reconstituted systems, the catalytic properties of the cytochrome components contained therein were still present, but they apparently lack the capacity to participate in the metabolism of $CCl_4$ (at least to the $\cdot CCl_3$ radical).

*Proof that the radical generated by the reconstituted systems was the trichloromethyl radical.* The radical generated by the $C_1$ and $C_2$ cytochrome fractions was demonstrated to be the $\cdot CCl_3$ radical by using $^{13}C$ as substrate in the reconstituted systems. The $^{13}C$-containing $\cdot CCl_3$ radical adduct which would be formed with PBN would have a $^{13}C$ atom in a position $\beta$ to the nitrogen atom of the spin-trap. As a result, the e.s.r. spectrum of the adduct would have 12 lines instead of the 6-line spectrum observed for the PBN radical adduct of $[^{12}C]\cdot CCl_3$ [2]. Figure 6G and 6H show that the e.s.r. signal obtained with the $C_1$ and $C_2$ fractions in reconstituted systems containing $[^{13}C]CCl_4$ and PBN had characteristics identical with that obtained previously for the metabolism of $[^{13}C]CCl_4$ *in vitro* and *in vivo* [2].

## DISCUSSION

The primary purpose of this study was to determine if the metabolism of $CCl_4$ to the $\cdot CCl_3$ radical could be achieved in reconstituted mixed function

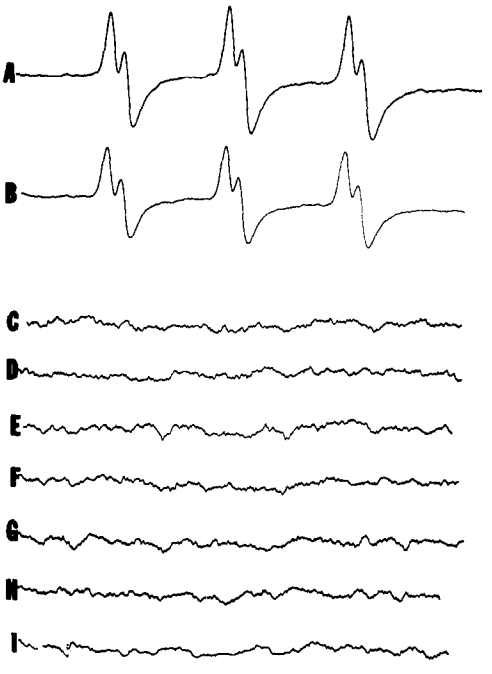


Fig. 4. Electron spin resonance spectra of a reconstituted rat liver mixed function oxidase system oxidizing NADPH in the presence of the spin-trapping agent, phenyl-t-butyl nitrone (PBN), and $CCl_4$. Purified phenobarbital-induced rat liver microsomal Fraction C, NADPH–cytochrome P-450 reductase, NADPH, $CCl_4$, and the spin-trapping agent, phenyl-t-butyl nitrone, were incubated and assayed for resonance signals as described under Methods. (A) Electron spin resonance spectrum of a microsomal incubation system containing 1.5 mg protein/ml, 0.14 M PBN in 0.05 M phosphate buffer (pH 7.4), 20 $\mu$l $CCl_4$ and an NADPH-generating system. (B) 3.16 nmoles cytochrome P-450 from Fraction C, 600 units of NADPH-cytochrome P-450 reductase, 0.14 M PBN, 20 $\mu$l $CCl_4$, and an NADPH-generating system (described in the legend of Fig. 6), final volume 1 ml. (C) Same as B, except that no NADPH–cytochrome P-450 reductase was added. (D) Same as B, except that no Fraction C cytochrome P-450 was added. (E) Same as B, except that cytochrome P-450 Fraction C was heat-denatured before addition. (F) Same as B, except treated with 1 mM *p*-chloromercuribenzoate. (G) Same as B, except that the system contained 0.1 mM *p*-chloromercuribenzoate. (G) Same as B, except that the system was bubbled with CO before addition of NADPH-generating system. (H) Same as B, except that 3.17 nmoles cytochrome P-450 Fraction A was used instead of Fraction C. (I) Same as B, except that 3.1 nmoles cytochrome P-450 Fraction B was used instead of Fraction C.

oxidase systems containing purified NADPH–cytochrome P-450 reductase and purified liver microsomal cytochrome P-450, or subfractions thereof. In addition, we wished to determine if metabolism of $CCl_4$ to the $\cdot CCl_3$ radical was a general property of the multiple forms of cytochrome P-450, or was limited to particular forms. The studies described in this report support the concept that both the reductase and cytochrome P-450 are required for the NADPH-dependent metabolism of $CCl_4$ to the $\cdot CCl_3$ radical. Addition of phospholipid was not required. The results clearly indicate that the 52,000 dalton form of cytochrome P-450 was involved in the reaction. Participation of the 54,000 dalton form was also likely but has not been proven. Other forms in the phenobarbital-induced rat did not appear to have this type of catalytic activity. The finding that the 52,000 dalton form was active in producing the $\cdot CCl_3$ radical from $CCl_4$ in reconstituted monooxygenase systems is consistent with the fact that this heme protein is induced by prior treatment of the rat with phenobarbital, and that phenobarbital treatment enhances the hepatotoxicity of $CCl_4$ [6]. The results are also consistent with the observation that the rapid loss of the 52,000 dalton cytochrome P-450 component is the first detectable change in the electrophoretic pattern of liver microsomal proteins following $CCl_4$ treatment in rats treated with either Aroclor 1254 or phenobarbital [1]. Since the highly reactive $\cdot CCl_3$ radical would be expected to abstract a hydrogen atom immediately in the vicinity of its formation (resulting in the production of chloroform), the mechanism of the specific cytochrome loss may be due either to a direct attack on the cytochrome, or to initiation of highly localized lipid peroxidation which could result in damage to the cytochrome. Possibly both processes are involved. Lipid peroxidation, if propagated for any significant period of time, would be expected to affect other P-450 cytochromes [19] if the multiple forms of these proteins are clustered in groupings within the membrane. Recent studies of DeGroot and Haas [20] indicate that lipid peroxidation may not be required for the destruction of cytochrome P-450 during $CCl_4$ metabolism since loss of the cytochrome occurred in microsomes incubated with $CCl_4$ and NADPH under anaerobic conditions. The assay for lipid peroxidation in those studies, however, was the determination of malondialdehyde (a minor product of lipid oxidative breakdown), and the observation of cytochrome P-450 loss in the absence of malondialdehyde formation does not eliminate the possibility that microsomal polyunsaturated lipid radicals ($L\cdot$) formed from hydrogen abstraction by $\cdot CCl_3$ in the absence of oxygen may be involved in the loss of the heme proteins.

Wolf *et al.* [21] could not detect the utilization of $CCl_4$ by microsomal systems under aerobic conditions, even though lipid peroxidation and NADPH oxidation were occurring. Under anaerobic conditions, they could detect $CCl_4$ utilization. However, we have observed $\cdot CCl_3$ formation under aerobic conditions *in vitro* as well as in the liver of intact animals [3] where, presumably, aerobic conditions exist. We interpret these seemingly contradictory results as being due to the capacity of the e.s.r. technique to detect very low concentrations of spin-trapped radicals as compared to the analysis of $CCl_4$ utilization by the gas chromatographic technique employed by Wolf *et al.* [21]. In addition, the low level of $CCl_4$ utilization under aerobic conditions may be a consequence of some influence of $O_2$ on properties of the cytochrome heme group since Wolf *et al.* reported that the metabolism of $CCl_4$ under anaerobic conditions in a reconstituted monooxygenase system is inhibited by both CO and $O_2$ [21]. Even in the presence of CO, however, we still observed significant $\cdot CCl_3$ production in CO-treated

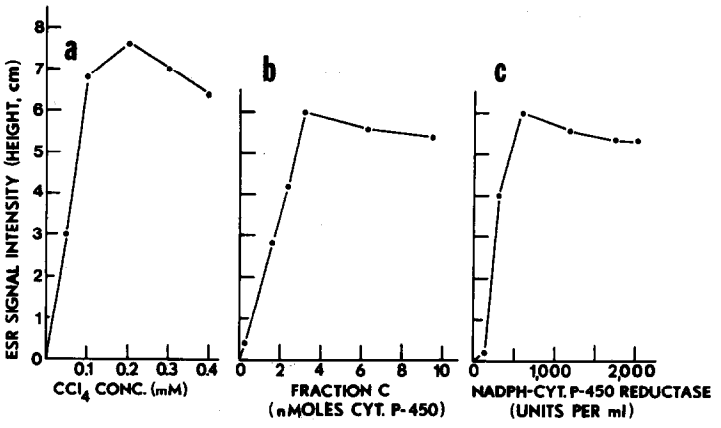


Fig. 5. Electron spin resonance signal intensities observed in reconstituted systems containing purified cytochrome P-450 Fraction C. Compositions of the systems were as follows (except for the particular component varied as indicated above): Fraction C (3 nmoles cytochrome P-450), NADPH–cytochrome P-450 reductase (600 units, 0.3 ml of an NADPH-generating system (5 mM glucose-6-phosphate, 0.3 mM NADP, 0.5 Kornberg units of glucose-6-phosphate dehydrogenase per ml of regenerating system), $CCl_4$ (20 μl/ml reaction system), and 0.14 M phenyl-t-butylnitrone, all in 0.05 M phosphate buffer, pH 7.4. The incubations were carried out for 15 min at 37°. The systems were placed in a Pasteur pipette and placed in the e.s.r. spectrometer cavity for measurement of signal intensity. Key: (a) effect of varying the concentration of $CCl_4$; (b) effect of varying the concentration of cytochrome P-450 Fraction C; and (c) effect of varying the amount of NADPH–cytochrome P-450 reductase in the system.

microsomes but not in reconstituted monooxygenase systems.

In our studies, the loss of total cytochrome P-450 continued for a period of at least up to 4 hr *in vivo* [1]. The changes in electrophoretic patterns of microsomal proteins that occurred after longer periods of incubation (2 hr or more) indicate that various proteins which are not in the molecular weight range of the P-450 cytochromes were also lost from the membrane, indicating widespread, but selective,

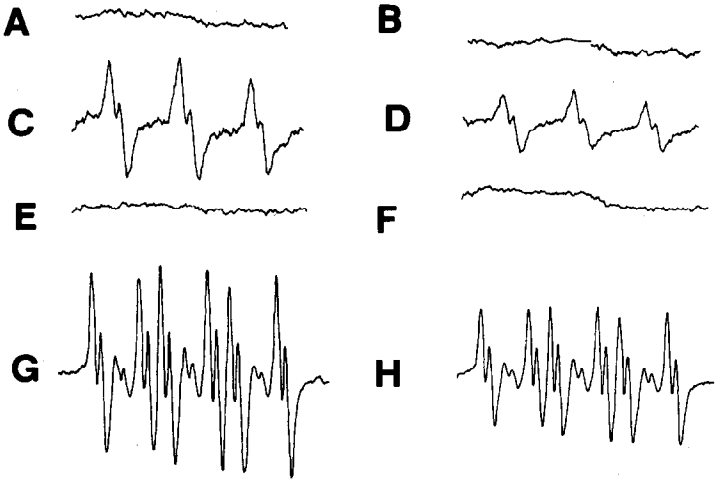


Fig. 6. Electron spin resonance spectra of reconstituted monooxygenase systems containing Fractions $C_1$ or $C_2$ in the presence of the spin-trapping agent, phenyl-t-butyl nitrone, and $CCl_4$. The incubation systems, experimental conditions, and analyses for e.s.r. signals are described under Methods. (A) 3.0 nmoles cytochrome P-450 Fraction $C_1$ (52,000 daltons), 600 units of NADPH–cytochrome P-450 reductase, 0.14 M phenyl-t-butyl nitrone, and 20 μl $CCl_4$. (B) 3.0 nmoles cytochrome P-450 Fraction $C_2$ (primarily 54,000 daltons), 600 units of NADPH-cytochrome P-450 reductase, 0.14 M phenyl-t-butyl nitrone, and 20 μl $CCl_4$. (C) Same as A, except that 0.3 mM NADPH was added. (D) Same as B, except that 0.3 mM NADPH was added. (E) 3.0 nmoles cytochrome P-450 Fraction $C_1$, 600 units of NADPH–cytochrome P-450 reductase, 0.14 M phenyl-t-butyl nitrone, and 20 μl $[^{13}C]CCl_4$. (F) 3.0 nmoles Fraction $C_2$, 600 units NADPH–cytochrome P-450 reductase, 0.14 M phenyl-t-butyl nitrone, and 20 μl $[^{13}C]CCl_4$. (G) Same as E, except that 0.3 mM NADPH was added. (H) Same as F, except that 0.3 mM NADPH was added.

molecular disorganization of the microsomal membrane subsequent to the initial attack on the P-450 cytochromes. With regard to the selectivity of loss, the reductase appeared to stay firmly anchored in the residual parts of the membrane even after very significant loss of other proteins.

In studies on the metabolism of 1,2,2-trichloroethane by reconstituted rat liver microsomal monooxygenase systems, there was an indication that a specific form or forms of cytochrome P-450 might be involved [22]. Specificity of various forms of cytochrome P-450 for certain substrates has been observed for other compounds. For example, of four different forms of rabbit cytochromes, only one can metabolize 2-aminoanthrene to a mutagenic compound (form 4), while still a different one (form 6) can metabolize benz[*a*]pyrene to a mutagenic substance [23]. Other forms of cytochrome P-450 show a high degree of, although incomplete, substrate specificity [24]. The mechanism of conversion of $CCl_4$ to $\cdot CCl_3$ is not known. It has been proposed that the cleavage of the carbon–chlorine bond is a function of NADPH–cytochrome P-450 reductase [25]. However, there is now considerable evidence to support the hypothesis that cytochrome P-450 is involved [21]. Our own studies described above indicate cytochrome involvement. In addition, the studies of Glende [26], in which there was 80% destruction of hepatic cytochrome P-450 by small doses of $CCl_4$ (which did not affect the activity of the reductase), showed protection of the animals from subsequent treatment with lethal doses of $CCl_4$. Also, Sipes *et al.* [27] have shown that lowering the level of the P-450 cytochromes by treatment of animals with allylisopropyl acetamide decreased the binding of labeled $CCl_4$ to both microsomal lipid and protein. Treatment of rats with allylisopropyl acetamide also decreases the formation of malondialdehyde by microsomes metabolizing $CCl_4$ *in vitro* [19]. The studies of other workers also support the involvement of cytochrome P-450 in $CCl_4$ metabolism [28]. The loss of absorbance of the reduced CO complex at 450 nm that occurs in microsomes of animals treated with $CCl_4$ is clearly due to the disappearance of the entire cytochrome polypeptide, as the accompanying paper demonstrates [1]. Since there are no detectable new bands in the slab gels in the molecular weight range below 52,000, it does not appear that loss of a peptide segment was responsible for the disappearance of the band. The early loss of the cytochromes, which initially appeared to be highly specific for the 52,000 dalton form, may have been the result of the detachment of these proteins from the membrane as a result of radical attacks on the membrane lipid matrix in the immediate vicinity of the proteins. Alternatively, a radical-mediated polymerization of the missing cytochromes may have resulted in a large molecular weight aggregate which did not enter the separating gel, at least as a discrete band. We are currently attempting to determine which, if either, of these possibilities may be the case, using the anti-52,000 dalton cytochrome P-450 antibody, among other approaches, as a means for detecting the protein. The significance of the generation of $\cdot CCl_3$ radicals from $CCl_4$ (and associated loss of the cytochrome required for their formation) to the biological consequences of $CCl_4$ exposure is enhanced by the known protective effects of free radical scavenging agents against the toxic action of $CCl_4$ [29]. It suggests that either the radicals *per se* or their further reaction products are essential for cell injury. Since the scavenging agents which provide protection against $CCl_4$ injury have been reported not to affect the binding of radioactively labeled $CCl_4$ to proteins and lipids in the endoplasmic reticulum [30], it would appear that formation of such adducts do not, in themselves, constitute the major insult to the liver cell.

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