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BINDING OF REACTIVE METABOLITES OF CCl_4 TO SPECIFIC MICROSOMAL PROTEINS*

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Abstract—The covalent binding of [^{14}C]carbon tetrachloride to microsomal proteins in rat liver microsomes under anaerobic conditions was investigated by SDS-polyacrylamide slab gel electrophoresis and fluorography. Most of the labeled proteins were observed in the molecular weight range of 52–61 kDa, indicating that cytochrome P-450 forms (EC 1.14.14.1) were labeled. Protein bands at the position of the NADPH-cytochrome P-450 reductase (78 kDa) (EC 1.6.2.4) and NADH-cytochrome b_5 reductase (33 kDa) (EC 1.6.2.2) also showed radioactivity. The fluorographic pattern of the protein labeling was cytochrome P-450-dependent, as was demonstrated by CO and metyrapone inhibition as well as by pretreatment of rats with inducing drugs such as 3-methylcholanthrene, benzo(*a*)pyrene, phenobarbitone and Aroclor 1254. Immuno-precipitation with a purified anti-P-450 immunoglobulin against cytochrome P-450 PB-B (52 kDa) of rat liver indicated that this protein contained about 10–20% of the total bound radioactivity in an average ratio of 0.8 mol [^{14}C]CCl_4-metabolite/mol cytochrome P-450 PB-B.

The reductive metabolism of CCl_4 and other polyhalogenated compounds (such as halothane, hexa- and pentachloroethane) under hypoxic conditions yields radicals [1–4]. The conversion of CCl_4 to the radical metabolites is catalyzed by the cytochrome P-450-dependent monooxygenase system [5, 6]. Other microsomal electron-donating enzymes like NADPH-cytochrome P-450 reductase or NADH-cytochrome b_5 reductase or cytochrome b_5 are not directly involved in the reductive metabolism of CCl_4 [7].

Recently two additional reactive intermediates of CCl_4, the trichlorocarbanion ($|CCl_3^{\ominus}$) and dichlorocarbene ($|CCl_2$) have been proposed as products of a second electron transfer to the CCl_3 radical [8, 9]. *In vitro* under anaerobic conditions about 80% of the CCl_4 is reduced to CCl_3 radicals and only 20% of CCl_3 to carbanions and carbenes [10]. Furthermore, a direct contribution of the carbanion or carbene on the CCl_4-induced covalent binding and lipid peroxidation is unlikely, because their stoichiometric conversion into stable reaction products, chloroform or CO, respectively, have been demonstrated [10]. Therefore, the toxic metabolites of the reductive dehalogenation of CCl_4 are the trichloromethyl- and the trichloromethylperoxy radical [11, 12].

The reactive radicals may also cause the inactivation of cytochrome P-450 *in vitro* and *in vivo* [13, 14] and bind to other proteins in the microsomal membrane. In this respect it has been reported that mainly microsomal proteins with molecular weights of 47–54 kDa are labeled by [^{14}C]CCl_4-metabolites [15, 16]. This suggests that mainly cytochrome P-450 species are the target proteins of the radicals.

These studies with [^{14}C]CCl_4 were undertaken to further characterize the nature of the [^{14}C]CCl_4-labeled microsomal proteins. Attempts were also made to measure the ratio of bound [^{14}C]CCl_4-metabolites and cytochrome P-450.

MATERIALS AND METHODS

Materials. [^{14}C]CCl_4 (3–5 mCi/mmol) was purchased from Amersham International (Amersham, U.K.). Molecular weight standards were from Serva (Heidelberg, F.R.G.). The antibody against cytochrome P-450 PB-B from rat liver was elicited in young male rabbits and purified by ammonium sulfate fractionation and DE-52 cellulose column chromatography and was a kind gift of Dr M. Noshiro [17].

Preparation of microsomes. Male Sprague–Dawley rats (100–150 g) were used after pretreatment with sodium phenobarbitone (PB)‖ (80 mg/kg body wt, i.p., daily for 3 days), Aroclor 1254 (500 mg/kg body wt, i.p., one single dose 4 days before killing), 3-methylcholanthrene (3-MC) (20 mg/kg body wt, i.p., daily for 2 days) and benzo(a)pyrene (BP) (20 mg/kg body wt, i.p. daily for 2 days). Liver microsomal fractions were prepared by the method of Frommer *et al.* [18]. Protein content was determined by the biuret method [19] and cytochrome P-450 by the procedure of Omura and Sato [20].

Incubations. The anaerobic incubation mixtures for [^{14}C]CCl_4 binding studies were prepared by mixing buffer, glucose-6-phosphate and $MgCl_2$ in a stoppered vessel and by bubbling N_2 through the solution

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‖ Abbreviations used: SDS, sodium dodecylsulfate; IG, immunoglobulin; PB, phenobarbitone; 3-MC, 3-methylcholanthrene; halothane, 1,1,1-trichlorobromochloroethane; metyrapone, 1,2-di-(3-pyridyl)-2-methyl-1-propanone.

for 10 min at 25°. Microsomes and glucose-6-phosphate dehydrogenase (EC 1.1.1.4.9) were then added and N_2 was flushed for an additional 5 min. NADPH was added and the reaction was started by [^{14}C]CCl_4 injection (0.1 M in methanol) 3 min later. Final concentrations of reagents were: 87 mM Tris–HCl, pH 7.6, 16 mM G–6–P, 12 mM $MgCl_2$, 0.5 mM NADPH, 1.5 U/ml G–6–PDH, 5 mg/ml microsomal protein and 1 mM CCl_4. The reaction was stopped by freezing aliquots in liquid nitrogen. Pure dioxygen was substituted for N_2 in this procedure to obtain assay mixtures with 100% O_2. Incubations with 1% O_2 were prepared by addition of a suitable amount of dioxygen to the stoppered reaction vessel (25 ml) that contained an anaerobic microsomal incubation mixture as described above. During the reaction the partial pressure of 1% oxygen was maintained in the incubations by vigorous shaking. Total volumes of the reaction mixtures were between 0.2 and 0.8 ml. The incubations were carried out at 25° for 20 min.

The covalent binding of [^{14}C]CCl_4 to microsomal proteins was measured according to the method of Uehleke *et al.* [21].

SDS-polyacrylamide gel electrophoresis and fluorography. Samples for electrophoresis were prepared by freeze-drying 0.1–0.2-ml aliquots of the microsomal incubations mixture. The residue was mixed with 100 μl of CCl_4 and freeze-dried again (high vacuum, cold trap at −180°) to displace not bound [^{14}C]CCl_4. Then the residue was redissolved in the electrophoresis buffer which contained 3 mg sodium dodecylsulfate (SDS)/mg protein and 0.01% dithiothreitol (DTT). Electrophoresis on SDS-polyacrylamide slab gels was carried out as described by Laemmli [22]. Gels were stained overnight in 0.1% Coomassie Brilliant Blue R-250, 45% methanol and 10% acetic acid. Gels for fluorographic detection of radioactivity were treated according to Bonner and Laskey [23] or similarly with EN-HANCER, NEN, U.S.A. The dry gels were exposed to Kodak X-Omat films at −75° for 5–7 weeks. The radioactivity of the protein bands on the gels was also determined by LSC counting directly. Each lane of the gels was cut in 3-mm slices and washed with CCl_4, buffer, HCl (0.01 M) and ethanol. The slices were incubated in 0.1 ml water for 2 hr and subsequently treated for 20 hr at 50° with 1 ml tissue solubilizer (Soluene-350, Packard, Frankfurt, F.R.G.). Finally, 10 ml scintillation cocktail (Dimilume-30, Packard, Frankfurt, F.R.G.) were added and after waiting for chemiluminescence to fade, radioactivity was measured by LSC counting. Counting efficiency was determined by the channel-ratio method. Quenching corrections were obtained by addition of a [^{14}C]standard to the solubilized samples.

Hemoproteins were detected in the polyacrylamide gels by the method of Thomas *et al.* [24].

Immunoprecipitation. A purified rabbit anti-P-450 PB-B immunoglobulin was used to isolate [^{14}C]CCl_4-labeled cytochrome P-450 by direct immunoprecipitation as described by Nabi *et al.* [25]. For the immunoprecipitation of cytochrome P-450 PB-B the incubation mixture was diluted with 50 mM potassium phosphate buffer, pH 7.4, which contained 20% glycerol and 0.9% NaCl and then solubilized by dropwise addition of sodium cholate to a final concentration of 1%. Aliquots (0.5-ml) containing 0.2–0.25 nmol of cytochrome P-450 were incubated with varying amounts of anti-P-450 immunoglobulin for 30 min at 25° and 16 hr at 4°. The immunoprecipitate was washed with immuno-reaction medium until the solution was free of radioactivity. The radioactivity of the immuno-precipitate could not be displaced by washing with CCl_4, buffer or ethanol. The precipitate was solubilized in 1 M NaOH for protein and radioactivity determination. The cytochrome P-450 content was determined as described by Noshiro *et al.* [26]. Counting efficiency and quenching correction were determined as mentioned above. For SDS-gel electrophoresis the immuno-precipitate was washed twice with electrophoresis buffer and then solubilized in 5% SDS.

Inhibition experiments with anti-P-450 PB-B immunoglobulin. Anaerobic incubations (0.15 ml) were prepared as described above except that the suspensions contained 2.2 mg microsomal protein/ml and 0.9% NaCl. Microsomes were preincubated with the antiserum 30 min before addition of NADPH and [^{14}C]CCl_4.

RESULTS

Anaerobic incubations of [^{14}C]CCl_4 with NADPH-reduced liver microsomal fractions produced reactive metabolites which can irreversibly bind to microsomal proteins. SDS-polyacrylamide slab gel electrophoresis and fluorography of a large number of such incubations resulted in radioactive binding patterns as shown in Fig. 1. The intensity of the radioactive bands was generally weak, therefore high concentrations of protein were separated on the gels and then exposed for 5–7 weeks to the films. Clear binding occurred mainly in the molecular weight region of 50–60 kDa to few microsomal proteins. The presence of NADPH was an absolute requirement for radioactive labeling.

Molecules combining with the catalytic site of cytochrome P-450 such as CO and metyrapone [1,2-di-(3-pyridyl)-2-methyl-1-propanone] inhibited the covalent binding pattern (Fig. 1). Carbon monoxide was the most effective inhibitor for the protein labeling in all examined microsomal fractions. Metyrapone was a good inhibitor in microsomal incubations of PB-, 3-MC- or Aroclor 1254-pretreated rats, whereas the inhibition by α-naphthoflavone was only significantly higher in microsomes of 3-MC-compared to BP- and Aroclor 1254-pretreated animals.

Since it is known that the amount of the cytochrome P-450 isoenzymes vary by pretreatment with inducing drugs, the radioactive binding pattern of [^{14}C]CCl_4 to microsomal proteins was determined after pretreatment of rats with different inducers. Protein bands were only weakly labeled with radioactivity after treatment with [^{14}C]CCl_4. The molecular weights of the labeled protein bands are listed in Table 1. Radioactive bands with molecular weights of about 52 and 54 kDa and of 58–61 kDa were present in microsomes of PB-, 3-MC-, BP- and Aroclor 1254-pretreated rats, whereas microsomes of control rats showed only a labeled protein band

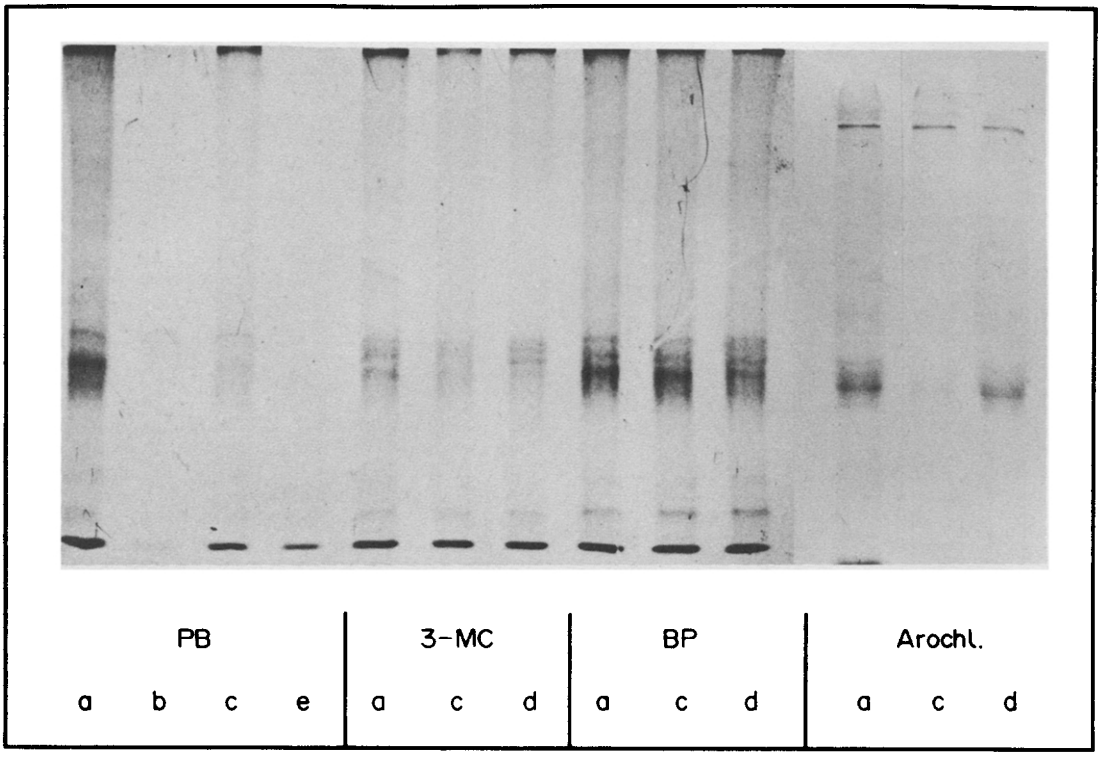

Fig. 1. Effect of various inhibitors on the fluorographic pattern of [^{14}C]CCl_4-labeled proteins of anaerobic NADPH reduced rat liver microsomes. Incubations were performed as described in Materials and Methods. The lyophylized microsomes (50 μg protein) were analyzed by SDS-polyacrylamide slab gel electrophoresis (10% polyacrylamide). The dry gels were exposed to films for 5–7 weeks at −75°. Microsomes of rats treated with different inducing agents were incubated (a) with 1 mM CCl_4; (b) in the presence of 100% CO, (c) 0.5 mM metyrapone or (d) 0.1 mM α-naphthoflavone, respectively. (e) Boiled microsomes were incubated with CCl_4. (PB, phenobarbitone; 3-MC, 3-methylcholanthrene; BP, benzo(a)pyrene; Arochl., Arochlor 1254).

Table 1. Effect of different inducing agents on the radioactive binding pattern of [^{14}C]CCl_4-labeled proteins in anaerobic NADPH reduced rat liver microsomes

Pretreatment	Mol. wts of the labeled proteins (kDa)						
Control	—	—	—	52	—	—	—
PB	27	33	49	52	54	58–61*	76–78
BP	27	33	—	52	54	58–61*	76–78
3-MC	27	33	—	52	54	58–61*	—
Aroclor 1254	—	—	49	52	54	58–61*	—

* In the molecular weight range between 58 and 61 kDa the labeled protein bands could not exactly be determined in different microsomal preparations. Incubation conditions were as in Fig. 1 and as described in Materials and Methods.

at about 52 kDa. Pretreatment with PB and Aroclor 1254 caused in addition a radioactive band at 49 kDa.

Benzidine staining of the SDS-slab gels failed to show the presence of heme in any of the labeled protein bands, although hemoprotein bands were detected on the gels of non CCl_4-treated microsomes in the molecular weight range of 52–61 kDa. These hemoprotein bands had the same molecular weights as the radioactive labeled proteins (data not shown).

A weak radioactive band between 76 and 78 kDa was found in microsomal fractions of PB-pretreated animals. This band had the same mobility as purified NADPH-cytochrome P-450 reductase. Radioactivity was also associated with two minor bands of about 33 kDa (molecular weight of the NADH-cytochrome b_5 reductase) and 27 kDa in all microsomal fractions except those of Aroclor 1254-pretreated and control rats. No radioactivity was associated with the protein band of cytochrome b_5 as was shown by SDS-gel electrophoresis on 15% polyacrylamide slab gels. Labeling occurred also at the tracking dye front and with protein aggregates with molecular weights more than 200 kDa, which hardly move into the gel.

Due to the high affinity of cytochrome P-450 for dioxygen it could be expected that the reductive metabolism of carbon tetrachloride and consequently also the covalent binding to microsomal proteins is suppressed by dioxygen in the incubation mixtures. However, in the presence of 1% dioxygen about 90% of the covalent binding to microsomal proteins were still formed whereas in the presence of 100% dioxygen the covalent binding in the molecular weight region of 50–60 kDa decreased to about 30% compared to anaerobic incubation (Fig. 2). The pattern of the labeled protein bands was however, similar in the presence of dioxygen and in anaerobiosis.

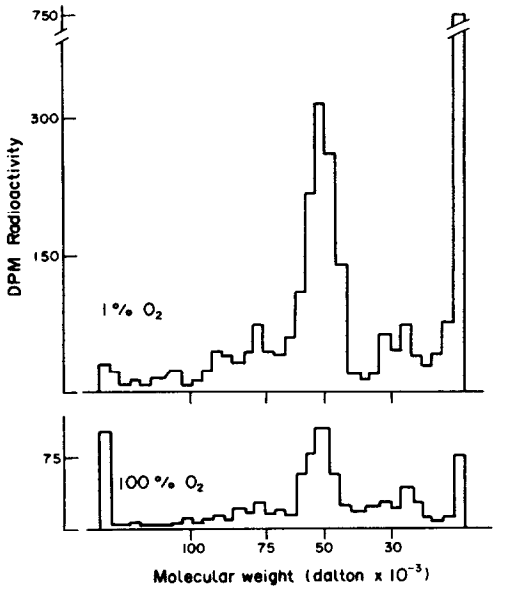

Fig. 2. Influence of dioxygen on the covalent binding of [^{14}C]CCl_4 to proteins in NADPH-reduced liver microsomes of PB-induced rats. The incubations were carried out as described in Materials and Methods but at room temperature under vigorous shaking in the presence of 1% of oxygen. The cytochrome P-450 concentration was 2.3 nmol/mg protein.

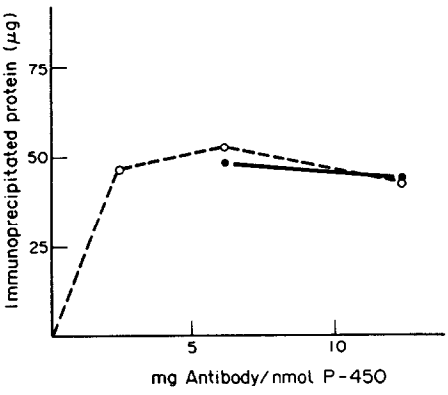

Fig. 3. Binding of anti-P-450 PB-B immunoglobulin to liver microsomes of PB-induced rats after treatment with [^{14}]CCl_4. The incubations with CCl_4 and anaerobic NADPH-reduced liver microsomes were carried out as described in Materials and Methods. The cytochrome P-450 concentration was 2.2 nmol/mg protein. Increasing amounts of anti-P-450 immunoglobulin were added to 0.5-ml aliquots of the solubilized microsomes (0.2–0.25 nmol cytochrome P-450) and incubated for 16 hr at 4° before separation of the precipitate.

The results of the SDS-polyacrylamide gel electrophoresis confirmed that radioactive labeling occurred mainly to microsomal proteins in the 50–60-kDa region. This is the molecular weight of several cytochrome P-450 forms. In order to obtain further evidence that cytochrome P-450 species are labeled by [^{14}C]CCl_4-metabolites, the cytochrome P-450 PB-B (52 kDa) was isolated from the reaction mixtures by means of immuno-precipitation with a purified rabbit anti-P-450 PB-B immunoglobulin. This anti-P-450 immunoglobulin was chosen because it has been demonstrated that mainly the cytochrome P-450 PB-B (52 kDa) of the liver is capable of generating the trichloromethyl radical from CCl_4.

The amount of protein used for the precipitation experiments was determined by addition of increasing antibody concentration to untreated or CCl_4-treated, solubilized microsomes of PB-pretreated rats (Fig. 3). The immuno-precipitation curves indicated that a ratio of about 4–7 mg anti-P-450 immunoglobulin to 1 nmol cytochrome P-450 could be applied for the immuno-precipitation experiments. This binding ratio was not changed after reaction of the NADPH-reduced microsomes with CCl_4.

In order to compare the extent of the radioactivity bound to cytochrome P-450 PB-B with that of total microsomal protein the covalent binding of [^{14}C]CCl_4 was measured both in the microsomal incubation mixtures and in the immuno-precipitates. The results are summarized in Table 2. Of the total radioactivity, 10–20% was associated with cytochrome P-450 PB-B which was precipitated by the anti-P-450 immunoglobulin (Table 2).

A comparison of cytochrome P-450 PB-B content with radioactivity in the immuno-precipitate indicated that about 0.8 mol CCl_4-metabolites were bound per 1 mol cytochrome P-450 PB-B in the precipitate (Table 2).

When the anti-P-450 PB-B immunoglobulin was present during the incubation of [^{14}C]CCl_4 with microsomes in the presence of NADPH under anaerobic conditions, the overall protein labeling decreased up to about 65% compared to control incubations with an unspecific rabbit immunoglobulin (Table 3).

DISCUSSION

The one-electron reduction product of CCl_4, the trichloromethyl radical, can further react with dioxygen to the trichloromethyl-peroxy radical. Both radicals are very reactive and can form chloroform [5], phosgene [27] or bind covalently to unsaturated lipids and microsomal proteins. The covalent binding and fluorography studies with anaerobic and aerobic reduced microsomes and CCl_4 indicate that the components of the monooxygenase enzyme complex, cytochrome P-450 species, NADPH-cytochrome P-450 (78 kDa) and NADH-cytochrome b_5 reductase (33 kDa) were labeled but not cytochrome b_5. The binding of the radicals at the reductases should not occur at a reactive group which is related to the catalytic site of the enzyme molecule, since during the reductive dehalogenation of CCl_4 the enzymatic activity of the NADH-cytochrome b_5- and NADPH-cytochrome P-450 reductase is not affected [28]. The radioactive protein aggregates which did not penetrate into the gel might be crosslinked protein molecules; and the radioactive bands at the tracking dye front of the gels might arise from CCl_3 radicals which

Table 2. Cytochrome P-450 content and radioactivity of immunoprecipitates obtained with anti-P-450 PB-B immunoglobulin and solubilized liver microsomes of PB induced rats

Immuno-globulin	Cyt. P-450 content of the immuno-precipitates (nmol)	(% total)	Radio-activity (dpm)	Molar ratio of bound CCl_4 to cyt. P-450 PB-B	% total protein bound radioactivity
1.25	0.024	11	212	0.86	10
1.25	0.027	11	373	1.29	14
1.25	0.030	12	215	0.83	10
1.25*	0.080	40	525	0.76	22
2.50	0.025	10	125	0.60	6
2.50	0.030	12	242	0.79	9
2.50	0.017	11	147	0.61	7
2.50*	0.076	38	376	0.57	16

* A rabbit anti-P-450 PB-B immunoglobulin preparation was used which was purified by immunoaffinity chromatography.
The assays were performed as described in Materials and Methods.

Table 3. Influence of antibodies on the covalent binding of [^{14}C]CCl_4 to proteins in anaerobic NADPH-reduced liver microsomes of PB-induced rats

Conditions	Ratio of the anti-body to cyt. P-450 (mg protein/nmol)	Microsomal protein (μg)	Radioactivity/ mg protein (dpm/mg protein)	Covalent binding (% control)
Control IG	3.7	75 (a)	18.450	100
Anti-P-450 PB-B	3.7	75 (a)	12.200	66
Control IG	9.6	129 (b)	7.194	100
Anti-P-450 PB-B	7.7	134 (b)	4.500	63

The assays were performed as described in Materials and Methods. (a) Cytochrome P-450 content was 3.1 nmol/mg microsomal protein. (b) Microsomes contained 2.2 nmol cytochrome P-450/mg microsomal protein.

bind to low-molecular-weight proteins and heme destruction products formed during the inactivation of cytochrome P-450.

Dioxygen, which competes with CCl_4 for the electrons of reduced cytochrome P-450, decreases the reductive metabolism of CCl_4 [7, 12] and therefore also the covalent binding of CCl_4-metabolites to microsomal proteins; the labeling pattern of the microsomal proteins between 50 and 60 kDa was not changed in the presence or absence of dioxygen. This suggests that the radicals react with the microsomal proteins under anaerobic and aerobic conditions by a similar mechanism in which lipid peroxidation is not involved.

The main form, cytochrome P-450 PB-B (52 kDa), can be induced by PB in rats at least 50-fold [29]. Noguchi *et al.* [30] have shown that the decrease in CCl_4 metabolism in CCl_4-treated rats is associated with a loss of the 52 kDa polypeptide. Consistent with these results a relative high labeling of the 52 kDa P-450-cytochrome by CCl_4 was found. Furthermore, the specific immunoglobulin against the cytochrome P-450 PB-B inhibited the covalent binding of the radicals to microsomal proteins by about 36%. A similar low inhibition of the covalent binding by this anti-P-450-immunoglobulin was found in the reductive metabolism of halothane, where this anti-P-450 immunoglobulin inhibited the reductive metabolism only to 20–30% compared to controls [3]. These results indicate that cytochrome P-450 PB-B is involved in the CCl_4-activation. The covalent binding and inhibition studies with the microsomes of rats pretreated with various inducers have demonstrated that other cytochrome P-450 forms were involved also, both in the activation of CCl_4 and in binding of the reactive intermediates formed. Thus, a participation of different cytochrome P-450 species in the formation of CCl_3 radicals cannot be excluded. This may explain why only 10–20% of the total protein-bound radioactivity was linked to cytochrome P-450-B (52 kDa) whereas the rest may be bound to P-450-A, P-450-C, P-450-D (48–56 kDa) [31], the NADH- and NADPH-reductase, polymerization products of cytochrome P-450 [30] and other microsomal proteins.

The immuno-precipitation of the radioactively labeled cytochrome P-450 PB-B by an anti-P-450 PB-B immunoglobulin demonstrates that about 1 mol CCl_4-metabolite is bound to 1 mol cytochrome P-450 (52 kDa) during the inactivation process of the enzyme. The labeling and inactivation of cytochrome P-450 could be due to an attack of the radicals at an exposed and reactive group at a protein site in the neighbourhood of the active center of the enzyme molecule. A candidate for such a reactive group at the cytochrome P-450 might be a cysteinyl group. Villarruel *et al.* [32] have found a preferential reaction of cysteinyl with CCl_3 radicals compared to other amino acids. The reaction of the radicals with the heme group of the enzyme may play also an important role in the inactivation process of cytochrome P-450 since the heme group is destroyed during the reaction of CCl_4 with cytochrome P-450 [13].

The work described here indicates that, in accordance with Noguchi *et al.* [30], mainly the 52-kDa P-450-cytochrome is responsible for the generation of the CCl_3 radical. Other cytochrome P-450 species [30] may also be capable of generating the radical but at a smaller rate. During this process the heme component is destroyed [13], the apoprotein is labeled and a radical-mediated polymerization of the cytochrome P-450 seems to occur [30]. The mechanism of this complex inactivation process, which is still not known yet, may start by a direct attack of the CCl_3 or CCl_3O_2 radical at the apoprotein or the heme group of the cytochrome P-450 and probably not by a lipid peroxidation product.

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