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STUDIES ON THE DESTRUCTION OF LIVER MICROSOMAL CYTOCHROME P-450 BY CARBON TETRACHLORIDE ADMINISTRATION

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Abstract—Carbon tetrachloride impairs the oxidative enzymes in liver microsomes by decreasing the amount of cytochrome P-450. The destruction is probably not mediated by an increase in lipid peroxidation, since ethanol which promotes lipid peroxidation does not decrease cytochrome P-450 or the metabolism of ethylmorphine. The decrease in cytochrome P-450 is not due to destruction of liver cells or to altered permeability of endoplasmic reticulum because NADPH cytochrome c reductase was not detected in plasma and was not decreased in liver microsomes. The solvent effect of CCl_4 probably does not account for the decrease in cytochrome P-450 because $CHCl_3$ and CH_2Cl_2 did not decrease the cytochrome. Our results are in accord with the view that CCl_4 acts through an active metabolite, but they do not necessarily imply that the active metabolite is formed by a cytochrome P-450 enzyme.

IT IS WELL known that the administration of carbon tetrachloride leads to various impairments in liver, including necrosis,[1] fatty infiltration[2] and decreased activity of microsomal enzymes that catalyze the oxidation of drugs.[3–5] It has become increasingly evident, however, that these impairing effects arise through diverse mechanisms. For example, prior administration of the antioxidants, tocopherol and *N,N*-diphenyl-*p*-phenylene diamine mitigates liver necrosis and fatty infiltration caused by CCl_4[6, 7] but does not prevent impairment of the microsomal enzyme systems that metabolize drugs.[7, 8] These findings thus suggest that impairment of the liver microsomal enzymes is not mediated by lipid peroxidation.

The present paper partially clarifies the mechanism through which carbon tetrachloride impairs the liver microsomal enzyme systems and some of the factors which affect the extent of impairment.

EXPERIMENTAL

Chemicals. Chloroform ($CHCl_3$) was obtained from J. T. Baker; methylene chloride (CH_2Cl_2) from Fisher Scientific and carbon tetrachloride (CCl_4) from Baker & Adamson.

Animals. Osborne–Mendel rats (180–240 g) were used to compare the effect of CCl_4 and ethanol on NADPH cytochrome c reductase activity, cytochrome P-450 content in liver microsomes and the kinetic constants of ethylmorphine *N*-demethylation.

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Osborn–Mendel rats were also used in studying the effect of CCl_4 in males and females. Unless otherwise noted, all other experiments were carried out with male Sprague–Dawley rats (180–220 g).

Treatment. Food was withdrawn 12 hr before administration of CCl_4, chloroform ($CHCl_3$) or methylene chloride (CH_2Cl_2), but water was available *ad libitum*. Undiluted CCl_4 was usually given p.o. at a dose of 2·5 ml/kg. When the effects of CCl_4, $CHCl_3$ and CH_2Cl_2 were compared, however, the three compounds were given p.o. at undiluted doses of 1·25 ml/kg, because some of the CH_2Cl_2-treated rats died at higher doses. The animals were sacrificed 3 hr after administration of the chlorinated hydrocarbons. In studies of CCl_4 toxicity in animals of different ages, the same conditions were used except that the rats were fed a Purina chow diet rather than fasted. The CCl_4 was administered to the newborn Sprague–Dawley rats with the use of a Hamilton microliter syringe adapted with polyethylene tubing (PE 10, Clay Adams Co., 0·024 in. o.d.). In order to facilitate the insertion of the tubing into the stomach, the tip was lubricated with some mineral oil.

Ethanol was given p.o. at a dose of 6 g/kg to fed rats in one experiment and to 12-hr fasted rats in another. The controls were either fed a Purina chow diet throughout the experiment or fasted for 12 hr before receiving an amount of glucose equivalent in calories to the ethanol administered. The animals were sacrificed 24 hr after ethanol administration.

Phenobarbital (80 mg/kg) was given to weanling Sprague–Dawley rats (30–50 g) once daily for 3 days. The CCl_4 was administered on the morning of the fourth day. Control rats received an equivalent aolume of saline i.p.

Enzyme preparation. The livers were homogenized in 4 vol. of ice-cold 1·15% potassium chloride with a Teflon-glass homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min in a Servall refrigerated centrifuge. The supernatant fraction was then centrifuged for 1 hr at 78,000 *g* in a Spinco model L preparative ultracentrifuge (*g* values were calculated for the center of the centrifuge tube). The microsomes were suspended in an ice-cold solution consisting of potassium chloride (1·15%) and Tris-HCl buffer (0·02 M, pH 7·4). Adrenal mitochondria and microsomes were separated according to the method of Schneider and Hogeboom.[9, 10]

Enzyme assay. In most experiments the incubation mixture contained 50 mM Tris buffer (pH 7·4), 5 mM $MgCl_2$, 8 mM sodium isocitrate, 0·33 mM NADPH, 0·36 unit/ml of isocitrate dehydrogenase (Boehringer & Soehne, 2·5 units/mg), about 2 mg/ml of microsomal protein and 1 mM ethylmorphine. In the experiments in which V_{max} and K_m were evaluated, the concentration of ethylmorphine was varied from 0·1 to 1·0 mM.[11]

The mixtures were incubated for 10 min at 37° in a Dubnoff metabolic shaker; the *N*-demethylation of ethylmorphine was estimated by measuring the amount of formaldehyde formed according to the method of Nash.[12]

NADPH cytochrome c reductase. The activity in microsomes was measured according to Phillips and Langdon.[13]

Cytochrome P-450 content. The amount of cytochrome P-450 was determined by the method described by Schenkman *et al.*[14]

Protein concentration. Protein concentrations were estimated by the method of Lowry *et al.*[15]

Statistics. The K_m and V_{max} values were calculated with a computer program.[11]

Two-way analysis of variance according to the method of Brownlee[16] was used to evaluate the significance of the data.

RESULTS

Effect of ethanol and CCl_4 on the kinetic constants for the N-*demethylation of ethylmorphine by liver microsomes.* Table 1 shows the effect of ethanol and CCl_4 on

TABLE 1. EFFECT OF ETHANOL AND CARBON TETRACHLORIDE ON THE KINETIC CONSTANTS FOR THE *N*-DEMETHYLATION OF ETHYLMORPHINE

Treatment*	K_m (mM)	V_{max} (mμmoles/mg/ 10 min)	Decrease in V_{max} (%)
Experiment 1			
Control (fed)	0·30 ± 0·02	203 ± 9	
Ethanol (fed)	0·35 ± 0·06	190 ± 10	6·5
Experiment 2			
Control (glucose)	0·50 ± 0·05	252 ± 30	
Ethanol (fasted)	0·45 ± 0·02	213 ± 20	15
Experiment 3			
Control	0·27 ± 0·03	184 ± 18	
CCl_4 (2 hr)	0·24 ± 0·03	102 ± 7†	44·3
CCl_4 (3 hr)	0·26 ± 0·06	79 ± 5†	57

* Ethanol was given orally at a dose of 6 g/kg to fed rats in the first experiment and to 12-hr fasted rats in the second; controls for the first experiment were fed *ad libitum*, whereas those for the second were treated with a calorically equivalent amount of glucose but not allowed access to food. The animals were sacrificed 24 hr after ethanol administration. CCl_4 was given orally at a dose of 2·5 ml/kg to 12-hr fasted rats; the animals were sacrificed 2 or 3 hr thereafter. Results are expressed as the mean of the values obtained with 5 animals ± S.E.

† The values are significantly different from control values ($P < 0·01$).

the kinetic constants for the *N*-demethylation of ethylmorphine by liver microsomes. The K_m values were not significantly changed 24 hr after ethanol administration to fed or to starved rats, nor were they changed 2–3 hr after CCl_4 administration. V_{max} values were decreased about 44 per cent at 2 hr and approximately 57 per cent at 3 hr after CCl_4 administration, but were not changed after ethanol administration. It is noteworthy that the K_m value was greater in the 36-hr fasted rats (control, experiment 2) than in the 12-hr fasted rats (control, experiment 3).

Effect of ethanol and CCl_4 on NADPH cytochrome c reductase and cytochrome P-450 content in liver microsomes. Table 2 shows the effect of ethanol and CCl_4 on NADPH cytochrome c reductase activity and P-450 content in liver microsomes. It was found that CCl_4 decreases the amount of cytochrome P-450 about 40 per cent, but that ethanol does not produce any significant change. NADPH cytochrome c reductase remained unchanged after CCl_4 treatment in the liver and was not detected in plasma. It is noteworthy that ethanol administered to fasted rats increased the activity of this enzyme in liver microsomes about 20 per cent.

TABLE 2. EFFECT OF ETHANOL AND CARBON TETRACHLORIDE ON NADPH CYTOCHROME c REDUCTASE AND CYTOCHROME P-450

Treatment*	P-450 (mμmoles/mg protein)	Relative amt.	NADPH cyt.creductase (mμmoles/min/mg)	Relative act.
Experiment 1				
Control	1·04 ± 0·02		72 ± 3	
CCl_4	0·62 ± 0·01†	0·60	72 ± 5	1·00
Experiment 2				
Control (glucose)	0·69 ± 0·04		92 ± 4	
Ethanol (fasted)	0·74 ± 0·04		110 ± 3†	1·20

* The experimental conditions were the same as those described in Table 1. Results are expressed as the mean of the values obtained with 6 animals ± S.E.
† The values are significantly different from control values ($P < 0·01$).

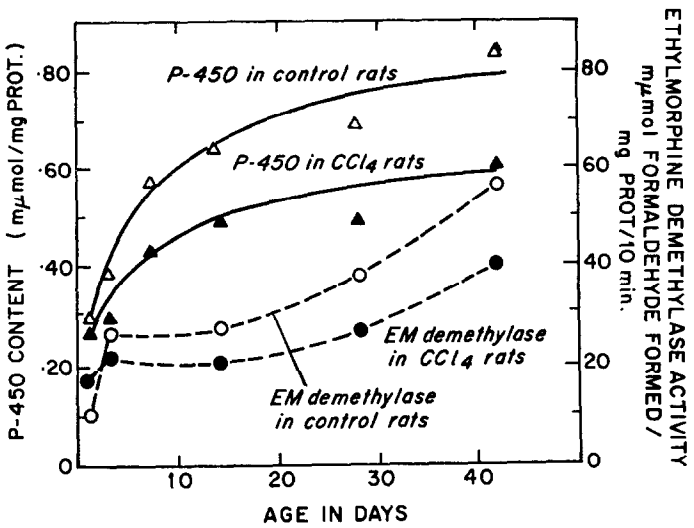
Effect of pretreatment with phenobarbital on the destruction of liver microsomal cytochrome P-450 by CCl_4. As shown in Table 3, phenobarbital administered to weanling rats almost tripled the activity of ethylmorphine *N*-demethylase and the amount of cytochrome P-450 in microsomes. The impairing effects of CCl_4 on both the demethylation of ethylmorphine and the amount of P-450 was significantly greater in the phenobarbital-treated animals than in the untreated controls.

TABLE 3. DESTRUCTION OF CYTOCHROME P-450 IN ANIMALS PRETREATED WITH PHENOBARBITAL FOR 3 DAYS

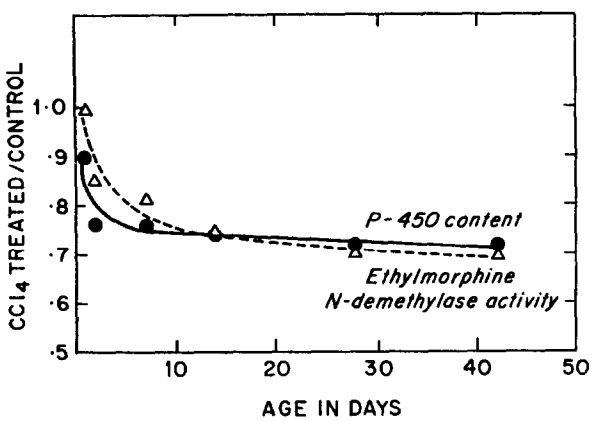
Treatment*	Ethylmorphine demethylation (mμmoles/mg/10 min)	Decrease (%)	Cytochrome P-450 (mμmoles/mg protein)	Decrease (%)
Control	61 ± 3		1·12 ± 0·07	
CCl_4	31 ± 3†	49	0·68 ± 0·05†	39
Phenobarbital	179 ± 10		2·81 ± 0·09	
Phenobarbital + CCl_4	62 ± 6†	65	0·68 ± 0·03†	76

*Phenobarbital was given i.p. to weaning rats once daily for 3 days at a dose of 80 mg/kg; the animals were killed on the morning of the fourth day. CCl_4 was given at a dose of 2·5 ml/kg to animals previously deprived of food for 12 hr. The animals were killed 3 hr thereafter. Results are expressed as the mean of the values obtained with 6 animals ± S.E.
† The values are significantly different from those of control or phenobarbital-treated animals ($P < 0·01$).

Effect of CCl_4 on liver microsomal cytochrome P-450 from fed rats of different age. We also studied the effect of CCl_4 in fed rats at ages ranging from 1 to 40 days old (Fig. 1, a and b). In newborn rats the destruction of cytochrome P-450 was very low and no impairment of *N*-demethylase activity was detected. The per cent of destruction of liver microsomal cytochrome P-450 by CCl_4 then reached a maximal value in 3-day-old animals and remained relatively constant in older animals, whereas the per cent of impairment of *N*-demethylase reached a maximal value in animals 3–14 days old (Fig. 1b).



(a)



(b)

FIG. 1 (a and b). Effect of CCl_4 on ethylmorphine demethylase and cytochrome P-450. Fed male rats of different ages received undiluted CCl_4 orally at a dose of 2·5 ml/kg. After 3 hr the animals were sacrificed and their livers were assayed for ethylmorphine demethylase activity and cytochrome P-450 content. Each point in the curves is the mean of duplicate analyses of pooled livers from several animals. The number of livers pooled in each case was: 20 for the 1-day-old rats; 15 for the 3-day-old rats, and 10 for the 7-, 14-, 28- and 42-day-old rats. In Fig. 1 (a), △———△ represents the amounts of cytochrome P-450 in liver microsomes of control rats; ▲———▲, the amount of cytochrome P-450 in liver microsomes of CCl_4-treated rats; ○———○, the *N*-demethylation of ethylmorphine by liver microsomes of control rats; and ●———●. the *N*-demethylation of ethylmorphine by liver microsomes of CCl_4-treated rats. In Fig. 1 (b), △———△ represents the CCl_4-treated/control ratio for *N*-demethylation of ethylmorphine and ●———● represents the CCl_4/control ratio for the amount of cytochrome P-450.

Comparative effect of CCl_4 on liver microsomal cytochrome P-450 from male and female rats. Table 4 shows the effect of CCl_4 on the *N*-demethylase and cytochrome P-450 in liver microsomes of male and female rats. The destructive effect is only slightly higher in males than in females and thus does not parallel the sex difference in the *N*-demethylation of ethylmorphine. The amount of cytochrome P-450 in liver microsomes was almost identical in male and female rats.

TABLE 4. COMPARISON OF THE EFFECTS OF CCl_4 IN MALE AND FEMALE RATS

Sex	Treatment*	Ethylmorphine demethylation (mμmoles/mg/10 min)	Decrease (%)	Cytochrome P-450 (mμmoles/mg protein)	Decrease (%)
Male	Control	100 + 4		1·04 ± 0·02	
	CCl_4	48 ± 4†	53	0·63 ± 0·02†	41
Female	Control	22 ± 1		1·16 ± 0·03	
	CCl_4	13 ± 0·6†	40	0·71 ± 0·04†	38

* CCl_4 was given orally at a dose of 2·5 ml/kg to animals deprived of food for 12 hr but having access to water *ad libitum*. The animals were killed 3 hr thereafter. Results are expressed as the mean of the values obtained with 6 animals ± S.E.

† The values are significantly different from those obtained with male or female controls ($P < 0·01$).

Comparative effect of CCl_4 on cytochrome P-450 content of different organs. At 3 hr after CCl_4 administration, there was a decrease of cytochrome P-450 not only in liver but also in adrenal microsomes (Table 5). In contrast, CCl_4 had little or no effect on the amount of cytochrome P-450 in kidney microsomes or adrenal mitochondria.

TABLE 5. EFFECT OF CARBON TETRACHLORIDE ON THE CYTOCHROME P-450 CONTENT OF DIFFERENT ORGANS*

Treatment	Cytochrome P-450 content (mμmoles/mg protein)							
	Liver microsomes	Decrease (%)	Kidney microsomes	Decrease (%)	Adrenal microsomes	Decrease (%)	Adrenal mito-chondria	Decrease (%)
Control	0·99 ± 0·07		0·15 ± 0·01		0·53 ± 0·05		1·23	
CCl_4	0·53 ± 0·03†	47	0·15 ± 0·01	0	0·33 ± 0·05†	37·7	1·17	4·9

* Results are expressed as the mean of the values obtained with 6 animals (for liver and kidney) and with 3 groups of 12 animals each for adrenal microsomes. The results for adrenal mitochrondria represent the values obtained after pooling adrenals from 12 animals per group. CCl_4 was given orally at a dose of 2·5 mg/kg to animals deprived of food for 12 hr. The animals were killed 3 hr thereafter.

† The values are significantly different from the control values ($P < 0·01$).

Comparison of effects of CCl_4, $CHCl_3$ and CH_2Cl_2 on ethylmorphine demethylation and cytochrome P-450 content in liver microsomes. Dingell and Heinberg[8] reported that at 24 hr after administration, CH_2Cl_2 caused no impairment of aminopyrine metabolism and $CHCl_3$ was less toxic than CCl_4. As shown in Table 6, $CHCl_3$ and

TABLE 6. COMPARISON OF THE EFFECTS OF CCl_4, $CHCl_3$ and CH_2Cl_2 ON ETHYLMORPHINE *N*-DEMETHYLATION AND CYTOCHROME P-450 CONTENT IN LIVER MICROSOMES*

Treatment	Ethylmorphine demethylation (mμmoles/mg/10 min)	Relative act.	Cytochrome P-450 (mμmoles/mg protein)	Relative amt.
Control	84 ± 4	1·00	1·08 ± 0·06	1·00
CCl_4	46 ± 5†	0·55	0·65 ± 0·05†	0·60
$CHCl_3$	109 ± 10†	1·30	1·16 ± 0·05	1·07
CH_2Cl_2	104 ± 13	1·24	0·99 ± 0·09	0·92

* Results are expressed as a mean of the values obtained with 5 animals ± S.E. The compounds were given orally at a dose of 1·25 ml/kg to animals previously deprived of food for 12 hr. The animals were sacrificed 3 hr after administration of the different compounds.

† The values were significantly different from control values ($P < 0·01$).

CH_2Cl_2 at 3 hr after administration failed to impair the *N*-demethylation of ethylmorphine or to decrease significantly the amount of cytochrome P-450 in liver microsomes. Indeed, there was a small but significant increase in enzyme activity after $CHCl_3$ administration and a small but not significant increase after CH_2Cl_2 administration.

DISCUSSION

In accord with the findings of Smuckler,[6] the present studies indicate that the administration of CCl_4 impairs the NADPH-dependent oxidative enzymes in liver microsomes by causing irreversible damage to cytochrome P-450 and not by acting as a competitive inhibitor. The mechanism through which CCl_4 decreases the amount of cytochrome P-450, however, remains unclear. Recknagel and Ghoshal[17–20] have postulated that peroxidation of microsomal lipids is a vector of CCl_4 hepatoxicity. Nevertheless, Castro *et al.*[7] reported that pretreatment of rats with potent antioxidants like *d,l*-tocopherol acetate and *N,N*-diphenyl-*p*-phenylenediamine at doses known to prevent liver injury does not prevent the destruction of cytochrome P-450 by CCl_4. Moreover, orotic acid administered to rats does not decrease the *N*-demethylation of ethylmorphine in male rats,[21] even though this compound is known to increase lipid peroxidation.[22] These findings thus suggested that the destruction of P-450 is not mediated by lipid peroxides. In support of this conclusion, the administration of ethanol, which is known to increase lipid peroxidation in liver microsomes,[23–26] did not decrease the amount of cytochrome P-450, and in fact caused a small but significant increase in NADPH cytochrome c reductase activity.

It seems unlikely that the decrease in ethylmorphine demethylase activity and cytochrome P-450 is caused simply by destruction of liver cells or endoplasmic reticulum. In such injuries, all the components of the microsomal membranes would be expected to decrease in parallel and NADPH-cytochrome c reductase would be expected to appear in plasma. Our experiments showed, however, that CCl_4 did not decrease the amount of NADPH cytochrome c reductase in liver nor increase the activity of this enzyme in plasma.

Although blockade of protein synthesis is known to occur shortly after CCl_4 administration,[27] it is unlikely that this effect accounts for the decrease in cytochrome P-450. The activity of the oxidative enzymes increases slowly after phenobarbital

administration, reaching a maximum only after 3 or 4 days, and decreases slowly after phenobarbital has been withdrawn; indeed, from such data Arias and Deleon[28] have suggested that the biologic half-life of the oxidative enzyme systems in phenobarbital-treated rats is about 2 days. Nevertheless, within 3 hr after CCl_4 administration more than 70 per cent of the cytochrome P-450 is destroyed in liver microsomes of phenobarbital-treated animals.

It is also unlikely that the decrease in cytochrome P-450 is mediated by proteolytic enzymes liberated from lysosomes, because impairment of these organelles would not be expected to occur before the fifth hour after CCl_4 administration.[27] Nevertheless, impairment of lysosomes may play an important role in the extensive destruction of cytochrome P-450 observed 24 hr after CCl_4 administration.[7]

It has been suggested that CCl_4 causes toxicities simply by being dissolved in lipoid membranes.[29] However, this solvent effect probably does not account for destruction of cytochrome P-450, because chlorinated hydrocarbons, such as $CHCl_3$ and CH_2Cl_2, do not destroy cytochrome P-450 *in vivo* within 3 hr after their administration. Moreover, the solvent effect would not account for our finding that CCl_4 causes greater destruction of cytochrome P-450 in the phenobarbital-treated rats than in control rats. In addition, CCl_4 caused almost no destruction of the cytochrome in newborn rats even though the maximum levels of CCl_4 in the liver of newborn rats are similar to those in the liver of adults.[30]

The finding that $CHCl_3$ and CH_2Cl_2 do not impair cytochrome P-450 *in vivo* during the first 3 hr suggests the possibility that an active metabolite is formed during the conversion of CCl_4 to $CHCl_3$, but not during the transformation of $CHCl_3$ and CH_2Cl_2 to CO_2.[31–33] According to this view, pretreatment of rats with phenobarbital enhances the formation of the active metabolite, which in turn leads to a greater destruction of cytochrome P-450. If an active metabolite of CCl_4 mediates the inactivation of cytochrome P-450, however, it must be formed by an enzyme having about the same activity in females as that in males for there was virtually no sex difference in the decrease in cytochrome P-450 or in the impairment of the enzyme that *N*-demethylates ethylmorphine. In this regard, it is noteworthy that Kato and Gillette[34] found that the magnitude of the sex difference in drug metabolism varies markedly with the substrate. Although there is a marked sex difference in the metabolism of hexobarbital and aminopyrine, there is almost no sex difference in the metabolism of aniline and zoxazolamine.

The formation of an active metabolite could also account for the age difference in the impairing effects of CCl_4. In newborn rats ethylmorphine is metabolized slowly and CCl_4 causes no destruction of cytochrome P-450. Accordingly, CCl_4 evokes almost no hepatoxic effects in newborn animals. The per cent of destruction of cytochrome P-450 caused by CCl_4 increases to a maximal value within 3 days and remains relatively constant thereafter. These findings thus suggest that an enzyme which converts CCl_4 to an active metabolite is lacking or poorly developed in the newborn rats, but rapidly increases in activity and is almost totally developed soon after birth. It is noteworthy that a variety of enzymes, such as glucose 6-phosphatase,[35, 36] glycerophosphate dehydrogenase,[37] phenylalanine transaminase and tyrosine transaminase,[38] are virtually inactive in the liver of newborn animals but rapidly develop during the first week of life.[35–38] It is therefore possible that CCl_4 is converted to an active metabolite by enzymes other than those which require cytochrome P-450.

During the past few years cytochrome P-450 has been found in the microsomal fraction of a number of tissues[39, 40] and in the mitochondria of the adrenal cortex.[41] It was therefore of interest to determine whether CCl_4 caused a decrease of cytochrome P-450 in other tissues as well as in liver. As shown in Table 5, within 3 hr after administration of CCl_4 the amount of cytochrome P-450 decreases in adrenal microsomes but not in kidney microsomes. These results support the view that CCl_4 exerts its action through the formation of an active metabolite and that the enzyme catalyzing the conversion is present in adrenal microsomes but not in kidney microsomes. Whether the impairment of adrenal microsomal cytochrome P-450 is related to CCl_4-induced adrenal necrosis,[42] however, remains to be investigated.

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