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SELECTIVE EARLY LOSS OF POLYPEPTIDES IN LIVER MICROSOMES OF CCl_4-TREATED RATS

RELATIONSHIP TO CYTOCHROME P-450 CONTENT*

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Abstract—Treatment of rats with carbon tetrachloride (CCl_4) resulted in early reproducible losses of either one or two specific polypeptides (depending on the inducing agent with which the animals had been treated) in the molecular weight range of the multiple forms of cytochrome P-450. The loss was correlated with a decrease in total cytochrome P-450 content in the microsome. The results of this study and those in the accompanying report indicate that CCl_4 was metabolized by a specific form of cytochrome P-450 (52,000 daltons), which was rapidly destroyed in the process. The early loss of this peptide occurred simultaneously with the previously demonstrated production of highly reactive trichloromethyl radicals ($\cdot CCl_3$). This polypeptide, which was shown to disappear from liver microsomes following treatment of rats with CCl_4, was demonstrated in the accompanying report to be the form of cytochrome P-450 specifically required for production of the highly reactive trichloromethyl radical in a reconstituted monooxygenase system.

Recent studies in this laboratory, focusing on the biological significance of lipid peroxidation, demonstrated unequivocally that the trichloromethyl radical ($\cdot CCl_3$) is formed during metabolism of carbon tetrachloride (CCl_4) in rat liver microsomes [1] and in rat liver *in vivo* [2, 3]. The studies on the microsomal polypeptides described in this report were undertaken because it appears that the earliest detectable events in the processing of CCl_4 by liver take place in the endoplasmic reticulum. On treatment of animals with an adequate dose of CCl_4, morphological changes in the endoplasmic reticulum are associated with evidence of concurrent lipid peroxidation [4, 5] and loss of enzyme activities [6, 7] as well as ultimately with fragmentation of that organelle [8]. Causal relationships between these events are not established, although it has been demonstrated that lipid peroxidation in liver microsomes results in a decreased cytochrome P-450 content [9]. In an effort to acquire more information concerning the relationships between CCl_4 metabolism, lipid peroxidation and damage to the endoplasmic reticulum, the investigations described in this report were pursued. They demonstrated that two hepatic microsomal polypeptides decrease in content during the early events which follow CCl_4 treatment. The accompanying report [10] demonstrates that the major polypeptide which was lost was a P-450 cytochrome that had the capacity to generate $\cdot CCl_3$ radicals in reconstituted monooxygenase systems while other forms did not. It had been postulated for a number of years that the metabolism of CCl_4 by liver tissue involved a free radical intermediate because (a) lipid peroxidation in the microsomal membrane accompanies the metabolism of CCl_4 *in vitro* [11, 12], (b) the liver damage caused by administering toxic levels of CCl_4 to animals can be mitigated by prior treatment of the animals with free radical scavenging agents [13, 14], and (c) conjugated dienes are detected in hepatic microsomal lipid extracts from CCl_4-treated rats [11, 12]. The effects of CCl_4 treatment on the hepatic microsomal polypeptides of interest in animals treated with phenobarbital are compared with similar studies on liver microsomes of animals induced with β-naphthoflavone or Aroclor 1254. The results of these studies and those in the following report support the concept that the manifestations of liver injury following exposure to CCl_4 depend initially on a specific form of inducible cytochrome P-450 that is selectively destroyed. The early destruction of this cytochrome may be associated with its ability to metabolize CCl_4 to the $\cdot CCl_3$ radical.

MATERIALS AND METHODS

Materials. The materials used in this study were obtained from the following sources: glycerol, Sigma Chemical Co., St. Louis, MO; Coomassie Blue, Canalco Inc., Elkhart, IN; *N,N'*-methylene-bis-acrylamide, Bio-Rad Laboratories, Richmond, CA; acrylamide, Miles Laboratories Inc., Elkhart, IN; *N,N,N'*-tetramethylethylenediamine and β-naphthoflavone, Aldrich Chemical Co., Milwaukee, WI; diethylaminoethyl cellulose (DE-52), Whatman Co., Aroclor 1254, Monsanto Chemical Co., St.

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Louis, MO; and phenobarbital, Merck Sharp & Dohme, Rahway, NJ.

Methods. Male, Sprague–Dawley rats (200–300 g) that had been maintained on a commercial rat ration were injected in one of the following ways: (a) phenobarbital intraperitoneally in 0.85% NaCl solution (75 mg/kg body weight for 4 days; (b) β-naphthoflavone intraperitoneally in 1.0 ml corn oil (80 mg/kg body weight, single dose); or (c) Aroclor 1254 intraperitoneally in 1.0 ml corn oil (300 mg/kg body weight, single dose). The respective controls were given vehicle only. Following such treatment, the animals were fasted overnight, and then administered 250 μl CCl_4/100 g body weight in 1.0 ml corn oil intragastrically by stomach tube. Two types of control animals were used in these studies: (a) rats treated with the same inducing agent, similarly fasted, but administered corn oil without CCl_4; and (b) rats not treated with the inducing agent and given corn oil without CCl_4. At different time intervals, the rats were decapitated and the livers removed. Liver microsomes were prepared by homogenizing the liver in 1.15% KCl (2:1, w/v) [15] and were subjected to centrifugation at 10,000 *g* for 15 min. The supernatant fraction was then centrifuged at 105,000 *g* for 1.0 hr. The pellets were resuspended in 1.15% KCl and centrifuged at 105,000 *g* twice. The final microsomal pellets were resuspended in 50 mM Tris–HCl buffer (pH 7.7) so that the microsomes from 1 *g* of liver were suspended in 1.0 ml of buffer. The protein concentrations of microsomal suspensions were determined by the method of Lowry *et al.* [16]. The concentration of total cytochrome P-450 in the microsomal preparations was determined by the method of Omura and Sato [17] from the CO difference spectrum of dithionite-reduced samples, using an extinction coefficient of 91 mM^{-1} cm^{-1}.

Polyacrylamide slab gel electrophoresis of solubilized microsomes was performed at 10° in the presence of sodium dodecylsulfate (SDS) using a Hoefer Scientific model SE-500 apparatus. This procedure was performed as described by Laemmli [18]. Prior to sample application, microsomal suspensions were diluted to 0.5 mg protein/ml with 0.06 M Tris buffer (pH 6.8) containing 1% SDS, 12% glycerol, 1.2% β-mercaptoethanol, and 0.001% bromophenol blue, and heated for 3 min at 100°. The separating slab gel (10 × 14 × 0.075 cm) contained 7.5% acrylamide. Electrophoresis was carried out at 12 mA/gel during stacking and at 20–25 mA/gel during separation. The gels were fixed, stained, and preserved, as described previously [19]. The apparent minimum molecular weights of the microsomal polypeptides of interest were estimated by comparison with pure protein standards of known molecular weights, which included NADPH–cytochrome P-450 reductase (79,000), bovine serum albumin (68,000), catalase (58,000), glutamate dehydrogenase (53,000), and ovalbumin (45,000). Designation of individual polypeptide bands with a particular molecular weight is based on the assignment of a value of 52,000 daltons for the primary phenobarbital-induced component as described by West *et al.* [15].

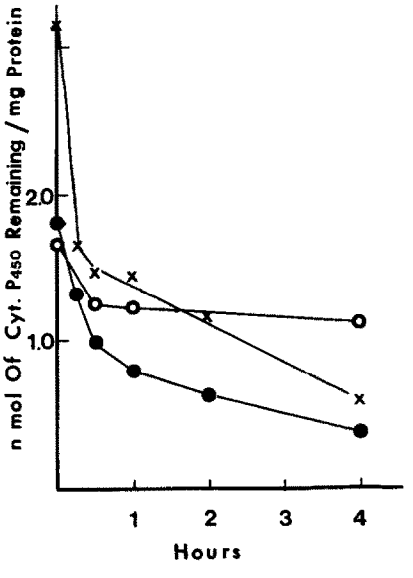

Fig. 1. Course of the decrease of total liver microsomal cytochrome P-450 in induced rats treated with carbon tetrachloride. Key: (●) rats treated with phenobarbital prior to CCl_4 administration; (×) rats treated with Aroclor 1254 prior to CCl_4 administration; and (○) rats treated with β-naphthoflavone prior to CCl_4 treatment. Treatment procedures and analyses of cytochrome P-450 content are described under Methods.

RESULTS

Figure 1 shows the time course of the decrease in the absorbance of the reduced CO complex of the total microsomal cytochrome P-450 between the time of administration of CCl_4 and 4 hr later. Both the phenobarbital- and Aroclor-treated animals showed a marked loss of absorbance at 450 nm, while the β-naphthoflavone-treated animals showed only about a 25–30% loss of total absorbance 4 hr after CCl_4 administration. The absorbance decrease was very rapid within the first hour and then declined more slowly thereafter. In terms of absolute loss, the Aroclor-treated animals displayed the greatest response (Fig. 1). The losses of total cytochrome P-450 per mg of microsomal protein 1 hr after the administration of CCl_4 were: Aroclor-treated rats, 1.69 nmoles; phenobarbital-treated rats, 1.0 nmoles, and β-naphthoflavone-treated rats, 0.43 nmole. Rats that had been treated with phenobarbital, Aroclor, or β-naphthoflavone were administered CCl_4, as described under Methods, and killed at various time intervals starting 15 min after administration of the halocarbon. Liver microsomes were prepared immediately, and the total microsomal proteins were subjected to slab gel electrophoresis as described under Methods. Figure 2 shows that, when animals were pretreated with phenobarbital, among the effects observed was the expected induction of the polypeptide corresponding to an apparent minimum molecular weight of 52,000 daltons (compare lanes C and D in Fig. 2). When the liver microsomes were prepared 15 min after administration of CCl_4, the band corresponding to the 52,000 dalton P-450 cytochromes had become less distinct (lane E) and

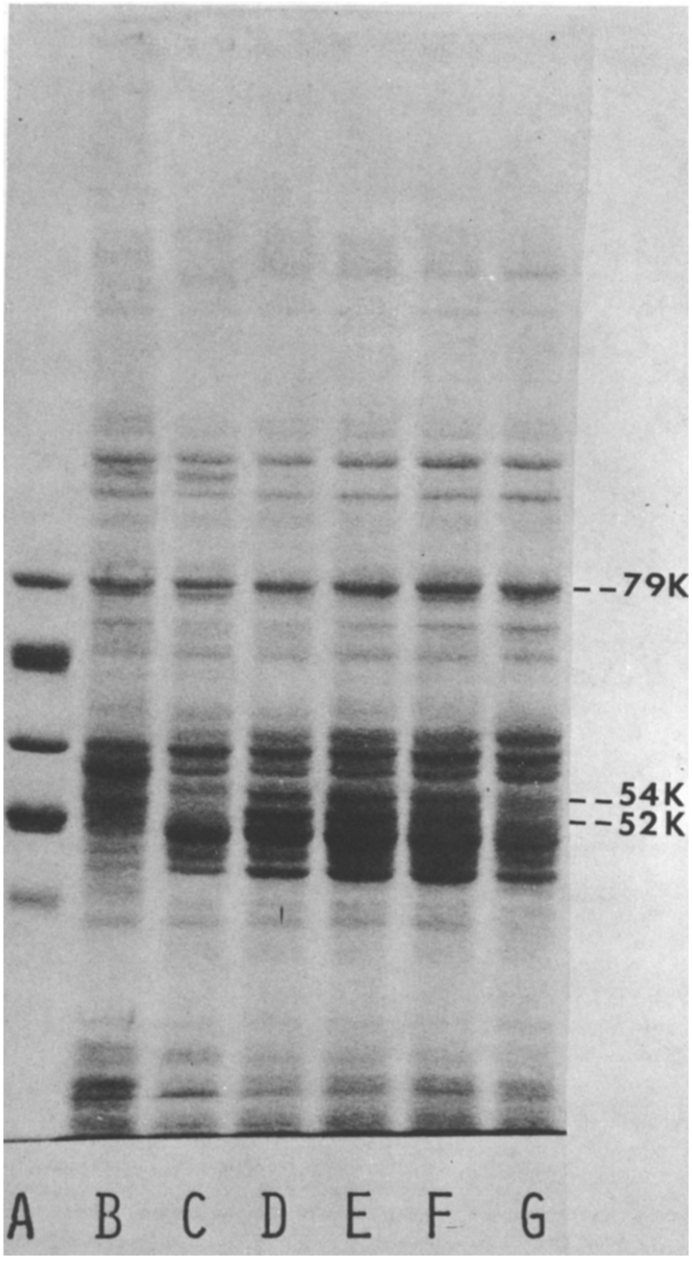

Fig. 2. SDS-polyacrylamide gel electrophoresis of liver microsomes from phenobarbital-induced rats treated with CCl_4 *in vivo*. Microsomes were isolated and prepared for electrophoresis as described under Methods. Key: (A) Protein standards (bottom to top): ovalbumin (45,000 daltons), glutamate dehydrogenase (53,000 daltons), catalase (58,000 daltons), bovine serum albumin (68,000 daltons), and NADPH–cytochrome P-450 reductase (79,000 daltons). Each of the following lanes had wells containing 10 μg of solubilized hepatic microsomal protein from: (B) β-naphthoflavone-treated rats, and (C) normal rats. The following lanes had wells containing 10 μg of solubilized hepatic microsomal protein from phenobarbital-induced rats that had been treated with CCl_4 (see Methods) and from which the microsomes were prepared at the following times after administering the halocarbon: (D) 0 min, (E) 15 min, (F) 30 min, and (G) 60 min.

after 30 min was considerably diminished (lane F). A component at 54,000 daltons also was beginning to diminish at 30 min but to a lesser extent (lane F). In the accompanying paper, the 52,000 dalton component was isolated and shown to be a cytochrome P-450 [10]. Other polypeptides in the cytochrome P-450 molecular weight range were still essentially unchanged at that time. Usually, by 1 hr after CCl_4 administration (lane G), both of the 52,000 and 54,000 dalton bands had essentially disappeared, and other polypeptide components both inside and outside of the molecular weight range of the P-450 cytochromes showed some decreases (lane G). The NADPH–cytochrome P-450 reductase band showed no discernible alteration throughout the period of analysis (Fig. 2, 79K component).

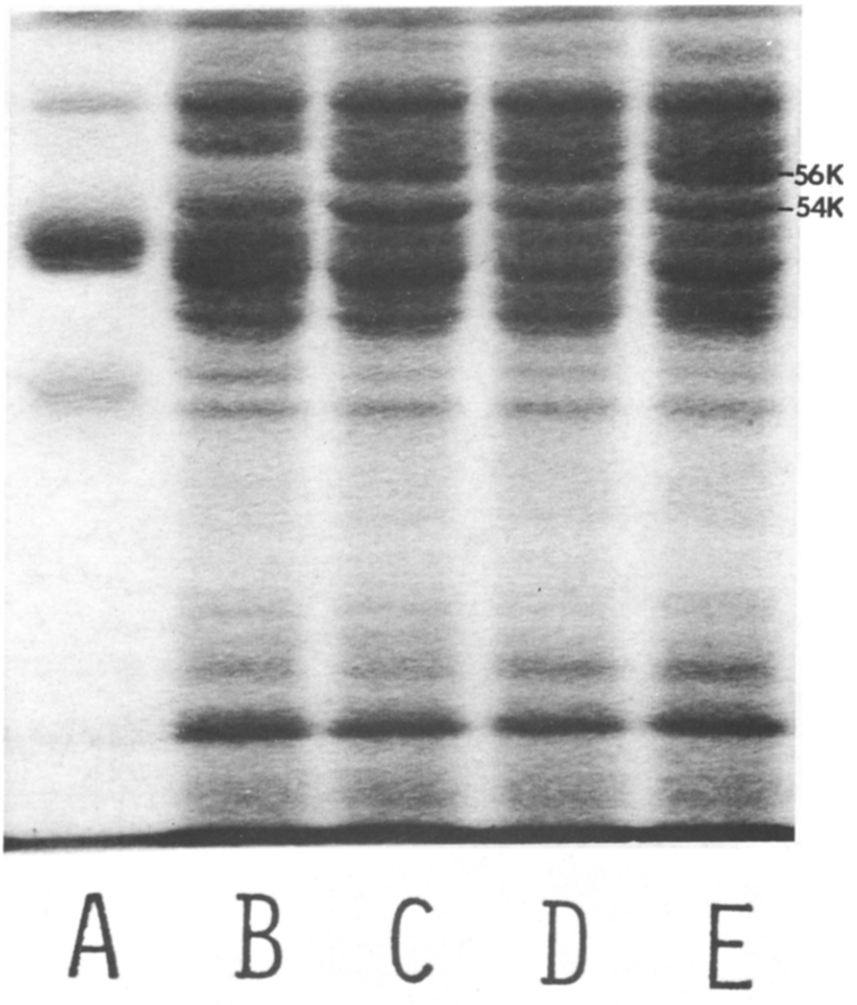

Fig. 3. Polyacrylamide slab gel electrophoretic pattern of polypeptides in the molecular weight range of the P-450 cytochromes in solubilized liver microsomes of β-naphthoflavone-induced rats. Key: (A) Protein standards (bottom to top): ovalbumin (45,000 daltons), glutamate dehydrogenase (53,000 daltons), and catalase (58,000 daltons); (B) normal rat; (C) β-naphthoflavone-induced rat: (D) β-naphthoflavone-induced rat treated with CCl_4 30 min before preparation of liver microsomes; and (E) as in "D" except that microsomes were prepared 1 hr after treatment with CCl_4.

The studies with animals treated with β-naphthoflavone, which induced polypeptide components with apparent minimum molecular weights of 54,000 and 56,000 daltons (compare lane B with lane C in Fig. 3), showed that observable selective loss in the polypeptides in the molecular weight range of the P-450 cytochromes for as long as 1 hr after CCl_4 treatment appeared to be limited primarily to the 54,000 dalton component (compare lane E with lane C). There was only a 25–30% decrease in total P-450 cytochrome content of the microsomes during that period (Fig. 1).

Animals treated with Aroclor 1254 prior to CCl_4 administration showed a marked induction of polypeptides with apparent minimum molecular weights of 52,000 and 56,000 daltons (Fig. 4; compare lanes B and C). When the Aroclor-treated rats were given CCl_4 and killed 15 min later, slab gel electrophoresis of liver microsomal proteins showed a decrease in the intensity of the 52,000 dalton polypeptide. After 1 hr (lane F) it was practically indistinguishable. The 56,000 dalton band, however, appeared to change very little. As indicated in Fig. 1, the total loss of P-450 cytochrome in Aroclor-induced animals

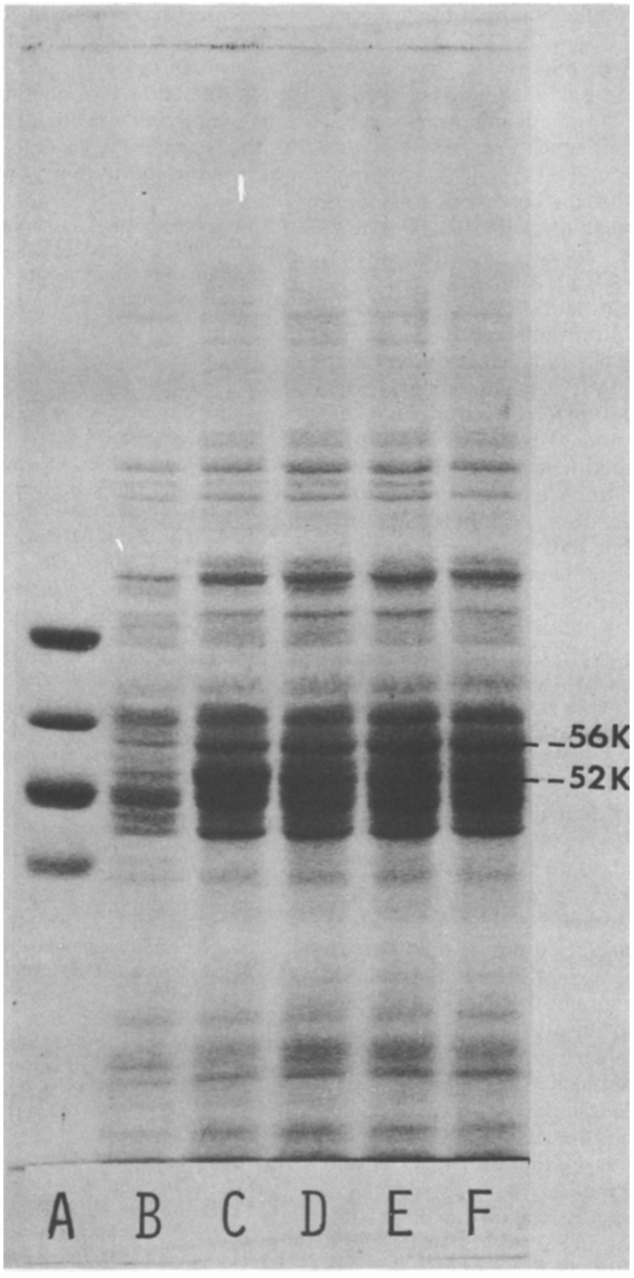

Fig. 4. Polyacrylamide slab gel electrophoretic pattern of peptides in solubilized liver microsomes from rats induced with Aroclor 1254. Electrophoretic procedures were as in Fig. 2. Key: (A) Protein standards (bottom to top): ovalbumin (45,000 daltons), glutamate dehydrogenase (53,000 daltons), catalase (58,000 daltons), and bovine serum albumin (68,000 daltons); (B) normal rats. The Aroclor-induced rats were administered CCl_4 after which the microsomes were prepared for electrophoresis at the following times after giving the halocarbon: (C) 0 min, (D) 15 min, (E) 30 min, and (F) 1 hr.

caused by treatment with CCl_4 was very substantial within 15 min, based on the absorbance of the reduced CO complex. Most of this decrease appeared to be associated with the loss of the 52,000 dalton polypeptide band.

DISCUSSION

The results of these studies indicate that the loss of the 52,000 dalton polypeptide present in the endoplasmic reticulum of the liver apparently determines the extent to which the cytochrome P-450 content can be decreased in that membrane following treatment with CCl_4. When comparing the results of prior induction of animals with phenobarbital or with β-naphthoflavone, it becomes evident that the forms of cytochrome P-450 induced are determining factors in the loss of P-450 hemoprotein. The microsomes of animals treated with either of these two inducing agents contained similar levels of total cytochrome

P-450 initially, but the phenobarbital-treated rats showed a loss of about 70% of the total cytochrome P-450 content in the liver microsomes within 4 hr while β-naphthoflavone-treated animals showed a loss of only about 30% of hepatic microsomal cytochrome P-450 within the same time period at the same dose level of CCl_4 (Fig. 1). These findings correlate with the fact that phenobarbital treatment produced a marked induction of the 52,000 dalton P-450 polypeptide (which appears to be the primary component affected by CCl_4 treatment).

β-Naphthoflavone treatment, on the other hand, increased the 54,000 dalton polypeptide (which, in the phenobarbital-induced rat liver microsome, appears to be destroyed by CCl_4 treatment to a lesser extent than the 52,000 dalton form) and also induced the 56,000 dalton component which is apparently resistant to the CCl_4 treatment in rats. The results indicate that the 52,000 and, to a lesser extent, the 54,000 dalton polypeptides are the microsomal proteins which are most labile to CCl_4. The observation that essentially the entire polypeptide moiety (shown to be a cytochrome P-450 in the accompanying report [10]) appears to be lost from the microsomal membrane in animals treated with CCl_4 may explain the finding by Guzelian and Swisher [20] that the formation of ^{14}CO from heme in rats pulse-labeled with 5-amino[5-^{14}C]levulinic acid was not increased when the animals were given CCl_4. Since CO production is considered to be a reliable assay for heme catabolism by animal tissues [21], these results mean that the heme groups (which have been shown to be degraded as a result of treating animals with CCl_4 [22]) must be broken down as postulated by Guzelian and Swisher [20] in a manner that does not produce CO. Because the CO produced during lipid peroxidation in microsomes does not appear to originate from heme [23], even though there is loss of cytochrome P-450 based on absorbancy measurements [9, 24], the decrease of microsomal heme in animals treated with CCl_4 without increased evolution of CO may be explained, at least in part, by the loss of the entire heme protein from the membrane. The heme group in the protein may either be released and reutilized or, as mentioned above, degraded by a non-CO-forming pathway. It is clear from these studies that it is not just the heme groups of the cytochromes that are lost but, as indicated by the slab gel studies, the polypeptide portion as well. However, there is a selective loss of proteins from the microsomal membrane since cytochrome b_5 remains essentially unchanged after CCl_4 treatment [25] and, as can be observed in Fig. 2, several polypeptide bands, including NADPH-cytochrome P-450-reductase, appear to remain attached to the microsomal membrane even after a major portion of the polypeptide components in the cytochrome P-450 range has been lost. Essentially the same results can be obtained *in vitro* by isolating microsomes from the three types of induced animals and incubating the microsomes with CCl_4 and NADPH for a period of 15–20 min (manuscript in preparation). This being the case, studies were undertaken to determine if the forms of cytochrome P-450 which appear to be most affected by CCl_4 metabolism initially are those which catalyzed the metabolism of the halocarbon to the reactive $\cdot CCl_3$ radical. These studies are described in the following report [10].

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