Effect of Ethanol on $CHCl_3$ Metabolism in Hepatic Microsomes from Osborne-Mendel Rats

Emanuela Testai,[1] Simonetta Gemma,[1] PierGiovanni Gervasi,[2] Silvia Menicagli,[2] and Luciano Vittozzi[1]

[1]Istituto Superiore di Sanita, Comparative Toxicology and Ecotoxicology Department, Biochemical Toxicology Unit, Rome; [2]Istituto di Mutagenesi e Differenziamento, Consiglio Nazionale delle Ricerche, Pisa, Italy

The treatment of Osborne-Mendel rats with ethanol in drinking water for 2 weeks resulted in a 3-fold increase of hepatic microsomal hydroxylation of both *p*-nitrophenol and aniline, two substrates considered highly selective for P4502E1. No other forms of P450 seemed to be affected. These results, confirmed by the immunoblot analysis of microsomal protein, showed an induction of P4502E1. The levels of total covalent binding to microsomal phospholipid due to $^{14}CHCl_3$ reactive intermediates in ethanol-pretreated microsomes were identical to those measured in microsomes from untreated rats at any pO_2. The distribution of radioactivity obtained after transmethylation of the adducts of $^{14}CHCl_3$ intermediates with microsomal phospholipids (PL) indicated that binding to fatty acyl chains (due to $\cdot CHCl_2$ radicals) increased with decreasing pO_2. On the contrary, the binding to polar heads due to phosgene decreased. The ethanol treatment did not affect binding to either PL moieties. These results indicated that, in our experimental conditions, the *in vitro* production of both oxidative and reductive intermediates of $CHCl_3$ in the liver of Osborne-Mendel rats were not influenced by ethanol consumption.—Environ Health Perspect 102(Suppl 9):25–30 (1994)

Key words: $CHCl_3$ activation, ethanol induction, P4502E1, liver microsomes, Osborne-Mendel rats, covalent binding

Introduction

The potentiation of $CHCl_3$-induced hepatotoxicity in rodents by ethanol (*1*) and other aliphatic alcohols (*2,3*), ketones and ketogenic compounds (*4–8*) is well known.

Various mechanisms have been proposed in order to explain this effect, such as increased absorption in the intestine (*9*), depletion of liver GSH (*10,11*), inhibition of hepatocellular regeneration and hepatolobular restoration (*3,7*), and hepatic hypermetabolism (*12*). However, the leading hypothesis refers to cytochrome P450 induction, responsible for an increase in $CHCl_3$ metabolism (*6,13–15*). The potentiating effects among alcohols and ketones differ significantly; this finding has been related to their qualitatively and quantitatively different capacities to induce various P450 isoenzymes (*16*). The involvment of P4502B1/2 has been evidenced (*14*) but much attention was drawn by P4502E1 (*15*) since it is the main ethanol- and acetone-inducible form (*17*) able to catalyze $CHCl_3$ biotransformation (*13*).

Until recently, studies on the association of the enhancement of $CHCl_3$-induced hepatotoxicity with its metabolism were related only to the detection of the $CHCl_3$ oxidation reactive intermediate, phosgene (*6,14*). No data are reported on the role of the reductive pathway, through which $CHCl_3$ is biotransformed to radical species ($\cdot CHCl_2$) (*18,19*), able to bind covalently to cellular structures (*20,21*).

Due to the social relevance of ethanol, to which most of human population can be exposed, we decided to study its interaction with $CHCl_3$, although this alcohol is not the most effective potentiator of $CHCl_3$ hepatotoxicity. We investigated the effect of ethanol pretreatment on the P450-dependent drug-metabolizing system in Osborne–Mendel hepatic microsomes. Then we investigated the effect produced by the same pretreatment on both oxidation and reduction pathways of $CHCl_3$ metabolism, using a $CHCl_3$ concentration (5 mM) at which both metabolic pathways are expressed (*21*).

Materials and Methods

Chemicals

(^{14}C)-Chloroform (3.6 mCi/mmole, radiochemical purity 99%) was obtained from New England Nuclear (Boston, MA). Unlabeled chloroform (IR purity) was from Merck (Darmstadt, Germany). Liquid scintillation cocktails Aqualuma and Lipoluma were purchased from Lumac Systems A.G. (Basel, Switzerland). Resorufin was obtained from Fluka (Buchs, Switzerland). 7-Ethoxycoumarin was from EGA-Chemie (Steinheim, Germany). Benzphetamine was supplied by Upjohn Co. (Kalamazoo, Michigan). Ethoxyresorufin and pentoxyresorufin were syntetized from resorufin by ethylation with ethyl iodide and by pentylation with pentyl iodide, respectively (*22*).

Enzymes and coenzymes were obtained from Boehringer GmBh (Mannheim, Germany). Nitrocellulose filters (0.45 µm), erythromycin, testosterone (T), 4-androsten-3,17-dione (17 OT), 16β-hydroxytestosterone (16β-OH), and corticosterone were supplied by Sigma Chemicals (St. Louis, MO). 2α-,2β-, 6α-, 6β-, 7α- and 16α-hydroxytestosterone (16α-OH) were obtained from the Steroids Reference Collection (DN Kirk, Department of Chemistry, Queen Mary College, London, England). Rabbit anti-rat P450

This article was presented at the IV European ISSX Meeting on Toxicological Evaluation of Chemical Interactions: Relevance of Social, Environmental and Occupational Factors held 3–6 July 1992 in Bologna, Italy

This work has been partially supported by the National Research Council within the frame of the Targeted Project, Clinical Applications of Oncologic Research, grant no. 9202310.PF39.

Address correspondence to Dr. Luciano Vittozzi, Istituto Superiore di Sanita, Viale Regina Elena, 299, I-00161 Roma, Italy. Telephone 39 6 4990. Fax 39 6 4440140.

2E1 polyclonal antibodies were purchased from Oxygene (Dallas, Texas).

All other analytical grade chemicals were obtained from common commercial sources.

Animals

Male Osborne-Mendel rats (180–200 g) were from Zentralinstitut fur Versuchstierzucht (Hannover, Germany). They were maintained on a 12-hr light cycle and provided food and water *ad libitum* for 1 week and then administered 15% (vol/vol) ethanol in drinking water for 2 weeks. Liver microsomal preparation were obtained as previously described (*23*).

Table 1. Effect of ethanol administration on monooxygenase activities in hepatic microsomes from Osborne-Mendel rats.

Enzymatic activity	Control microsomes	Ethanol-treated microsomes
P450[a]	0.6 ± 0.08	0.54 ± 0.11
b_5[a]	0.14 ± 0.01	0.16 ± 0.03
APND[b]	5.3 ± 0.91	4.7 ± 0.6
BZND[b]	4.9 ± 0.46	5.7 ± 0.80
ErND[b]	0.64 ± 0.08	0.50 ± 0.13
AnOH[b]	0.49 ± 0.12	1.10 ± 0.21
pNPH[b]	0.49 ± 0.006*	1.51 ± 0.008*
ECOD[b]	0.55 ± 0.13	0.83 ± 0.15
EROD[b]	0.11 ± 0.03	0.11 ± 0.02
PROD[c]	7.6 ± 2.5	6.0 ± 1.0

[a]nmole/mg proteins. [b]nmole/min/mg proteins. [c]pmole/min/mg proteins. Abbreviations: P450, cytochrome P450; b_5, cytochrome b_5; APND, aminopyrine *N*-demethylase; BZND, benzphetamine *N*-demethylase; ErND, erythromycine *N*-deethylase; AnOH, aniline hydroxylase; pNPH, *p*-nitrophenol hydroxylase; ECOD, 7-ethoxycoumarin-*O*-deethylase; EROD, ethoxyresorufin *O*-deethylase; PROD, pentoxyresorufin-*O*-depentylase. *Statistically significant values ($p<0.001$).

Table 2. Hydroxylation of testosterone by liver microsomes of control and ethanol-treated Osborne-Mendel rats.[a]

Metabolites	Control microsomes	Ethanol-treated microsomes
6α-OH	0.0012 ± 0.007	0.018 ± 0.01
7α-OH	0.028 ± 0.01	0.025 ± 0.01
6β-OH	0.16 ± 0.04	0.12 ± 0.03
16α-OH	0.31 ± 0.11	0.34 ± 0.09
16β-OH	0.014 ± 0.006	0.010 ± 0.004
2α-OH	0.25 ± 0.05	0.22 ± 0.03
2β-OH	0.032 ± 0.008	0.024 ± 0.007
17-OT	0.33 ± 0.06	0.25 ± 0.05
Total	1.13 ± 0.29	1.01 ± 0.23

[a] Results are expressed as nmole/min by mg protein. Values represent the mean ± standard deviation of four experiments performed with different microsomal preparations. Incubations were carried out at 37°C for 15 min with 1 mg/ml microsomal proteins.

Biochemical Assays

Microsomal protein content was determined by the method of Oyama and Eagle (*24*), using bovine serum albumin as a standard. Cytochrome P450 and cytochrome b_5 (b_5) were measured by the method of Omura and Sato (*25*). The activities for the *N*-demethylation of aminopyrine (APND), benzphetamine (BZND), and erytromycine (ErND) were assayed by measuring the formation of formaldehyde (*26*). Aniline hydroxylase (AnOH) and *p*-nitrophenol hydroxylase (pNPH) were determined according to Ko et al. (*27*) and Reinke and Moyer (*28*). The 7-ethoxycoumarin-*O*-deethylase (ECOD) activity was assayed by the method of Aitio (*29*). Ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-*O*-depentylase (PROD) activities were determined by measuring the formation of the corresponding hydroxy products (*30*).

Testosterone hydroxylase was assayed according to an HPLC method as described by Platt et al. (*31*).

Lipids were extracted according to Folch et al. (*32*).

Gel Electrophoresis and Immunoblotting

SDS-PAGE was carried out using the discontinuous system of Laemmli (*33*), using a 1.5-mm thick gel with 3 and 7.5% acrylamide in the stacking and separation gel. Proteins were transferred from the slab gel to the nitrocellulose filters, following the method of Towbin et al. (*34*). Immunodetection of P4502E1 was performed using rabbit polyclonal antibodies.

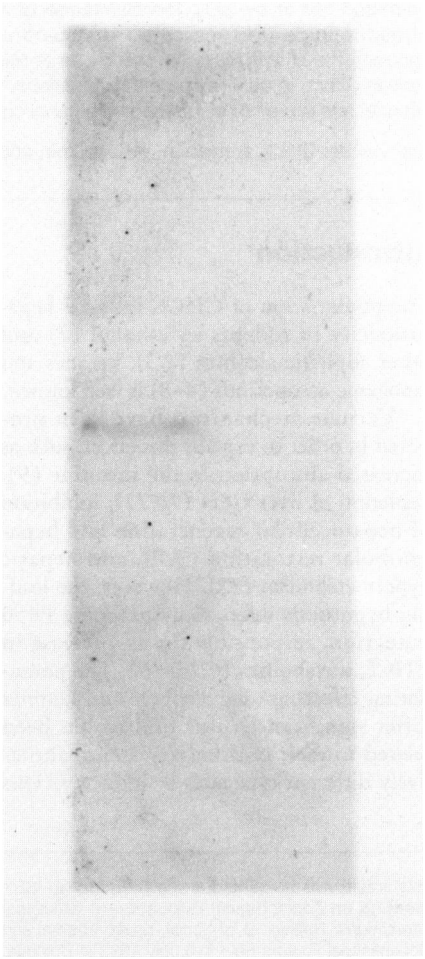
In Vitro Activation of ^{14}C-Chloroform

The standard incubation mixture contained microsomal protein (2 mg/ml), G6P (2 mM), $MgCl_2$ (2 mM) G6P-dehydrogenase (1 U/ml), EDTA (1 mM), NADP (0.2 mM) and ^{14}C-chloroform 5 mM in 50 mM Tris-HCl buffer, pH7.4. When anoxic conditions were required, the incubation mixture also contained an oxygen scavenging enzyme system. Mixture to be incubated under hypoxic (about 1% pO_2) or anoxic conditions were flushed with ultrapure N_2 for 20 min; a mixture of O_2 N_2 (5:95) was flushed for 20 min when incubations were to be carried out at 5% pO_2- A detailed description of the procedure was reported previously (*20*).

Covalent Binding of ^{14}C-Chloroform Metabolites

Covalent binding of ^{14}C-label to microsomal lipid was measured after 20 min incubation according to the method of Uehleke (*35*), with minor modifications (*20*). The regioselective binding of ^{14}C-chloroform metabolites to microsomal phospholipids (PL)-polar heads and/or fatty acyl chains was determined after the acid catalyzed transmethylation of PL-adducts, as described in detail by De Biasi et al. (*36*).

Briefly, the lipid extract was dissolved in 1 ml of anhidrous methanol: benzene: H_2SO_4 (75:25:1), and vigorously shaken for 1 hr at 70°C. The reaction mixture was cooled in an ice bath, then 2 ml petroleum ether (bp 40°–70°) and 1 ml 0.26M K_2HPO_4 were added. After 10 min vigorous shaking, the mixture was centrifuged (3000 rpm, 10 min). The lower aqueous



A B

Figure 1. Immunoblot analysis of hepatic microsomes from control (lane A) and ethanol-treated rats (lane B). Microsomes (12 μg protein loaded in each lane) were subjected to SDS-PAGE electrophoresis followed by immunoblotting.

layer, containing the hydrophylic polar heads, was transferred into a plastic vial containing 17 ml Aqualuma. The upper organic phase, containing the PL fatty acid methyl esters, was washed twice and then a 2-ml aliquot was transferred into a plastic vial containing 10 ml Lipoluma.

Calculations

Data obtained with control and ethanol treated microsomes were compared by means of the Student's *t*-test.

Results

Effects of Ethanol on Hepati~ Drug Metabolizing Enzymes

The effect of ethanol administration on hepatic cytochrome P450 and b_5 content and some monooxygenase activities is shown in Table 1. With respect to microsomes from control animals, the ethanol treatment resulted in a 2.4- and a 3-fold increase of the oxidation rates of *p*-nitrophenol and aniline, two substrates considered to be highly selective for P4502E1. All the other monooxygenase activities, as well as the amount of both P450 and cytochrome b_5, did not exhibit any significant difference between control and ethanol-treated rat liver microsomes.

To investigate more specifically the effect of ethanol administration to Osborne-Mendel rats on the constitutive P450 isozymes, the metabolism of an endogenous substrate, such as T was studied. Results presented in Table 2 show that no significant differences were present between control and ethanol-treated hepatic microsomes either in the total level of T-hydroxylation or in the production of any specific T-metabolite, including 16β-OH and 17 OT, which are associated, respectively, with P450 2B1/2 (the major PB-inducible P450 isoenzyme) and P450 2C11 (the most relevant constitutive P450 form in the rat liver).

The immunoblot analysis of microsomal protein Figure 1 using rabbit anti-rat P4502E1 polyclonal IgG, evidenced the absence of any reaction in control microsomes for lane A, whereas a band is present in lane B where ethanol-treatred rat liver microsomes were loaded.

Effect of Ethanol on Chloroform Metabolism

The levels of total covalent binding to microsomal phospholipids due to $^{14}CHCl_3$ reactive intermediates in ethanol-pretreated Osborne-Mendel rat liver microsomes were almost indentical to those measured in microsomes from untreated rats at any tested pO_2 (Figure 2).

With the transmethylation of the PL-adducts of Osborne-Mendel rat liver microsomes, it is possible to selectively quantitate the production of oxidation and reduction intermediates of $CHCl_3$ metabolism, which exhibit a typical regioselectivity in their attack to PL. Indeed, while $^{\bullet}CHCl_2$ radicals, reductively produced from $^{14}CHCl_3$, preferentially bind to PL fatty acyl chains (FC), the major product of $CHCl_3$ oxidation, phosgene, has in PL polar heads (PH) its main target (*36*). In Figure 3, radioactivity associated to PH, expression of $CHCl_3$ oxidation, measured in control and in ethanol-treatred Osborne-Mendel rat liver microsomes is shown. It

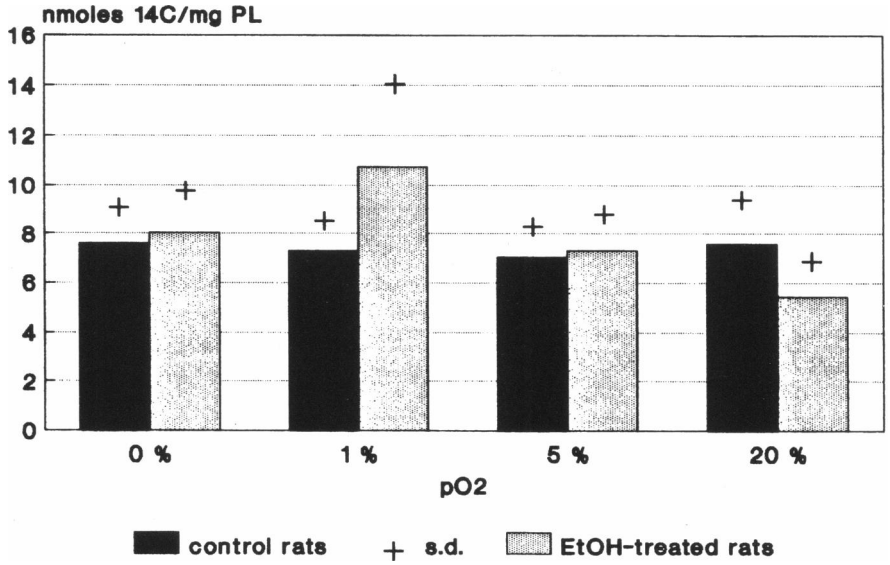


Figure 2. Oxygen concentration dependence of *in vitro* covalent binding of $CHCl_3$ metabolites to liver microsomal phospholipids. Incubations were carried out at 5 mM $^{14}CHCl_3$ with control (filled bars) and ethanol-treated (dotted bars) Osborne-Mendel rats hepatic microsomes. Values represent means and standard deviations calculated from three different microsomal preparations.

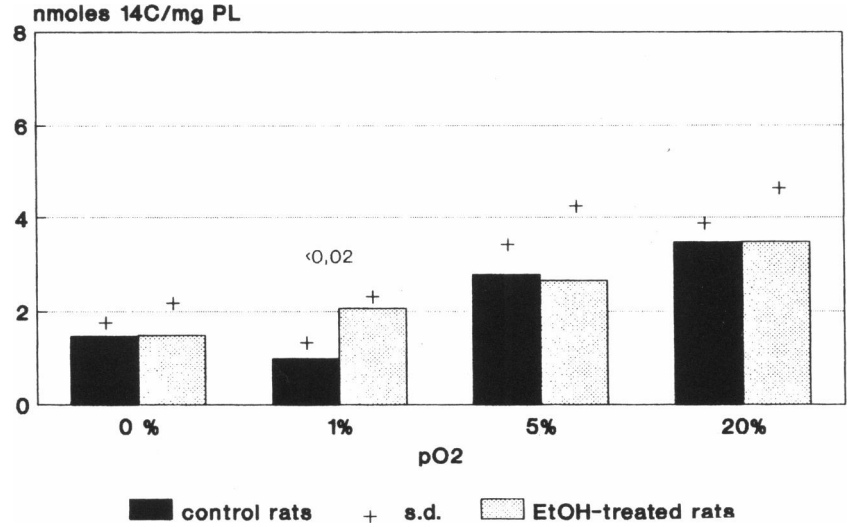


Figure 3. Effect of ethanol-pretreatment on *in vitro* covalent binding of $CHCl_3$ metabolites to liver microsomal PL polar heads. Incubations were carried out at 5 mM $^{14}CHCl_3$, at different O_2 tensions both with control (filled bars) and ethanol treated (dotted bars) Osborne-Mendel rat hepatic microsomes. Values represent means and standard deviations calculated from three different microsomal preparations. For the statistically significant difference between control and treated microsomes, the level of *p* is reported.

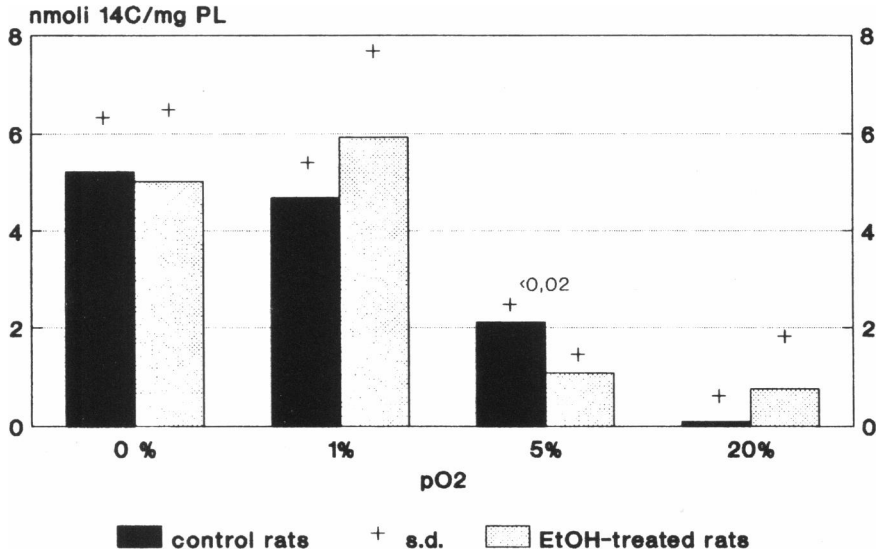


Figure 4. Effect of ethanol-pretreatment on *in vitro* covalent binding of $CHCl_3$ metabolites to liver microsomal PL fatty acyl chains (for details see Figure 3).

appears that binding to PH increased on increasing pO_2, but no differences were present between control and treated microsomes; only values measured at 1% pO_2 exhibited some degree of statistical significance. The binding to FC, considered as an index of $CHCl_3$ reduction in control and treated microsomes is shown in Figure 4. The opposite dependence on pO_2 with respect to PH was evidenced: the levels of radioactivity associated to FC decreased on increasing pO_2. Moreover, levels measured both in control and in treated microsomes were almost identical. The slight statistical significance of the difference between values at 5% pO_2 was not considered relevant. As a consequence, the relative contribution of the two different $CHCl_3$ metabolites to the almost similar levels of PL total covalent binding measured in different oxygenation conditions (Figure 2), markedly varied with pO_2 (Figures 3 and 4). Indeed, the ratios of radioactivity associated to PH versus radioactivity to FC (PH/FC ratios) were 0.28, 0.21, 1.34, and 38.5 in control microsomes. The ratios were 0.31, 0.36, 2.77 and 4.65 in ethanol treated microsomes, at increasing pO_2 from 0 to 1, 5 and 20%, respectively.

Discussion

Similar to previous findings with rabbits, (*37,38*) hamsters (*39*), and different strains of mice and rats (*40,41*), the present results clearly indicate that the oral administration of ethanol to Osborne-Mendel rats, resulted in the induction of P4502E1. In fact, the significant increase of the hepatic P4502E1-linked monooxygenase activities was confirmed by the immunodetection in microsomes from ethanol-treated rat liver, of a band corresponding to P4502E1. That band was absent in hepatic microsomes from control Osborne-Mendel rats.

No other forms of P450 seemed to be significantly affected by ethanol pretreatment, as demonstrated by the unchanged metabolism of either exogenous and endogenous P450 substrates.

Previous works evidenced that both P4502B1/2 and P4502E1 are involved in $CHCl_3$ metabolism, which can therefore be affected by pretreatment with PB, alcohols and ketones (*6,13–15,42*).

Although our pretreatment procedure resulted in the induction of P4502E1, in hepatic microsomes from Osborne-Mendel rats, no substantial quantitative changes were detectable in chloroform metabolism, which is expressed as $^{14}CHCl_3$-derived reactive intermediates covalently bound to microsomal PL (Figure 2).

Moreover, considering the typical regioselectivity in the attack to PL exhibited by oxidation and reduction intermediates of $CHCl_3$ (*36*), it appeared that ethanol was also uneffective in qualitatively modifying the pattern of $CHCl_3$ metabolism (Figures 3 and 4). Indeed, the two pathways are similarly expressed in control and ethanol-treated microsomes.

The relative contributions of P450 2B1/2 and P450 2E1 appear to be dependent on the substrate concentration. Indeed, it has been suggested (*42*) that at low chloroform concentrations (about 0.1 mM) its metabolism i catalyzed mainly by P450 2E1 (*13,15,42*), while P450 2B1/2 may be significantly responsible for $CHCl_3$ activation at a higher haloform concentration of 5 mM (*14*). Data on the effects of ethanol on chloroform metabolism at high substrate concentration were only based on the detection of chloroform oxidation products (*14*). Our data indicate that both the oxidative and the reductive metabolism of $CHCl_3$ are not affected by P450 2E1 at 5 mM $CHCl_3$, a concentration at which the two pathways are expressed (*21*).

One of the most relevant features of the $CHCl_3$ metabolism is that the oxygenation of the incubation mixture was of major importance in determining the oxidative and/or reductive nature of $CHCl_3$ activation (*21*). In this paper, we show that at 1 and 5% pO_2, representing the physiological range of O_2 tensions typical of the liver (*43*), the two pathways are concurrently present. It appeared also that in our *in vitro* conditions the shift from oxidation to reduction occurred just in this range Of pO_2. Indeed the relative magnitude of the binding to PH and FC was reversed mostly between 5 and 1% pO_2 (Figures 3 and 4).

Rats treated with ethanol showed a higher rate of O_2 consumption in the liver than control rats (*44*). This phenomenon may increase the state of physiological hypoxia of the liver and suggests that the consequent alteration of the delicate balance between the oxidative and the reductive activation of $CHCl_3$ may concur in the potentiation of $CHCl_3$ toxicity.

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