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# Cytochrome P450 reductase-mediated anaerobic biotransformation of ethanol to 1-hydroxyethyl-free radicals and acetaldehyde

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**Abstract**

The ability of cytochrome P450 reductase to metabolize ethanol (EtOH) to acetaldehyde (AC) and 1-hydroxyethyl free radicals (1HEt) in anaerobic media was studied. Determination of AC was made by GC-FID analysis of the head space of incubation mixtures. The formation of 1HEt was established by GC-MS analysis of the adduct formed between the radical and the spin trap PBN. Results showed that pure human P450 reductase is able to biotransform EtOH to AC and 1HEt in a NADPH-dependent process under an oxygen-free nitrogen atmosphere. Pure FAD in the presence of NADPH was also able to generate AC and 1HEt from the alcohol. Anaerobic incubation mixtures containing either rat liver microsomes or pure nuclei were also able to biotransform EtOH to AC and 1HEt in the presence of NADPH. These processes were inhibited by antibody against rat liver microsomal P450 reductase. Results suggest that semiquinone forms of the flavin in P450 reductase may biotransform EtOH. These reactions might be of some significance in tissues where the P450 reductase is present in the absence of specific forms of cytochrome P450 known to be involved in EtOH metabolism (e.g. CYP2E1). However the toxicological significance of this enzymatic process remains to be established. 

*Keywords:* Cytochrome P450 reductase; Anaerobic biotransformation; Ethanol; Acetaldehyde; 1-Hydroxyethyl free radical

## 1. Introduction

In the course of recent studies from our laboratory, the presence in liver nuclei of anaerobic enzymatic pathways of biotransformation of ethanol to acetaldehyde was reported. This biotransformation process required the presence of NADPH as cofactor and it was significantly enhanced by the simultaneous presence of FAD in the incubation mixture (Castro et al., 1998). These results prompted us to postulate that cytochrome P450 reductase might be involved in this pathway

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of ethanol biotransformation (Castro et al., 1998). Previous studies employing reconstituted systems evidenced that P450 reductase is able to oxidize ethanol under air in the presence of NADPH and in the absence of cytochrome P450 in an iron stimulated process, involving the formation of reactive oxygen species including hydroxyl radicals (Ohnishi and Lieber, 1978; Winston and Cederbaum, 1983, 1986). In those studies, the authors did not attempt to verify whether an anaerobic process might also occur. In the present study, we describe the strictly anaerobic possibility of pure P450 reductase to bioactivate ethanol to 1-hydroxyethyl free radicals and acetaldehyde and the possibility for this pathway to participate in liver microsomal and nuclear anaerobic biotransformation of alcohol.

## 2. Materials and methods

### 2.1. Chemicals

The polyclonal human liver microsomal P450 reductase (10.0 mg/ml) and the antibody against rat liver microsomal P450 reductase were from Gentest Corp., USA. Glucose-6-phosphate dehydrogenase, from yeast, was from Boehringer, Germany. Isocitric dehydrogenase (ICDh) from porcine heart, as well as $NADP^+$, NADH, FAD, ethanol-$d_5$ and *N-tert*-butyl-α-phenylnitrone (PBN) were from Sigma (St Louis, MO). All the other chemicals used were of the best available quality. Nitrogen (ultra high purity) was from AGA (Argentina) and was further deoxygenated by bubbling through a solution containing 0.05% 2-anthraquinone sulfonic acid sodium salt and 0.5% $Na_2S_2O_4$ in 0.1 N NaOH.

### 2.2. Animals

Non inbred Sprague–Dawley male rats (240–280 g) were used. The animals were starved for 12–14 h before sacrifice. Water was available ad libitum. Animals were killed by decapitation and their livers were rapidly excised and processed.

### 2.3. Isolation of nuclei

Highly purified nuclei were obtained as previously described (Castro et al., 1989, 1990; Díaz Gómez et al., 1999). Briefly the liver homogenate in 0.25 M sucrose in TKM buffer pH 7.5 (50 mM Tris–HCl, 3 mM $MgCl_2$ and 1 mM KCl) was passed through a 100-mesh nylon cloth and centrifuged at $1000 \times g$ for 20 min. After washing the pellet twice by resuspending with 0.25 M sucrose–TKM and centrifugation at $1000 \times g$ for 5 min, the crude preparation was resuspended in 2.2 M sucrose–TKM, layered on 2.3 M sucrose–TKM and centrifuged for 20 min at $80\,000 \times g$. The pellet was gently rinsed with 0.25 M sucrose–TKM and then resuspended in 2.2 M sucrose–TKM and the ultracentrifugation step was repeated. Finally, the pellet was washed with 1.0 M sucrose–TKM (centrifuged at 5 min at $2000 \times g$) and then with 0.25 M sucrose–TKM. The purity of nuclei was assessed on the basis of their lack of activity of marker enzymes for mitochondria (isocitric acid dehydrogenase), for cytosol (lactic dehydrogenase) and by electron microscopy in order to obtain a definitive assessment of the purity of nuclear preparations. These criteria were recently considered as the most suitable to assess that nuclei were free of detectable contamination from other organelles (Rickwood et al., 1997).

The hepatic microsomal fractions were isolated by the procedure already reported (Castro et al., 1997).

### 2.4. Biotransformation of ethanol to acetaldehyde by human cytochrome P450 reductase (P450 Red)

#### 2.4.1. Incubation mixture

A 3-ml reaction mixture consisted of: human P450 reductase (0.1 mg prot./ml), 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM $MgCl_2$ and 0.28 M ethanol in 300 mM $K_2HPO_4$ buffer pH 7.4. The incubation vial was thoroughly purged by passing oxygen-free nitrogen previous to ethanol. All reactants were purged with nitrogen and added to the incubation mixture through a septum in order to keep oxygen-

free atmosphere conditions. The reaction mixture was incubated for 1 h at 37°C under $N_2$ except when indicated. The reaction was stopped by placing the flasks on ice. Determination of acetaldehyde was as follows: After adding 1 ml of saturated NaCl solution, samples were kept at 40°C for 10 min and acetaldehyde was measured in an aliquot (100 μl) of the head space by GC-FID. Chromatographic conditions were: column Poraplot Q, 25 m × 0.53 mm i.d., (Chrompack, Netherlands), temperature 140°C, injection port temperature 150°C, FID: 200°C.

### *2.5. Inhibition of rat liver microsomal or nuclear ethanol metabolism to acetaldehyde with antibody against rat liver microsomal NADPH P450 reductase*

#### *2.5.1. Incubation mixture*

Liver microsomes (40 μg prot./ml) or liver nuclei (4.5–5 mg prot./ml), anti-rat NADPH P450 reductase serum (100 μl), NADPH generating system, 0.28 M ethanol in 300 mM $K_2HPO_4$, pH 7.4 for the case of microsomes or 0.25 M sucrose in TKM buffer, for the case of nuclei, was incubated under $N_2$ for 1 h at 37°C. The antiserum was pre-incubated with microsomes or nuclei at room temperature for 30 min. A control reaction was run using normal serum. Acetaldehyde was measured as described above.

### *2.6. 1-Hydroxyethyl radical generation in the FAD-NADPH model system*

In order to evaluate the ability of the redox system constituted by FAD and NADPH to generate free radicals from ethanol, the spin adduct of the 1-hydroxyethyl (1HEt) radical was generated in a chemical system, essentially as previously described by our laboratory (Castro et al., 1997). In this case, the incubation mixture contained: 1 mM FAD, 9.4 mM PBN, 0.28 M ethanol and NADPH generating system in 300 mM $K_2HPO_4$ buffer pH 7.4. The reaction mixture was incubated for 1 h at 37°C under $N_2$. Control samples performed in the absence of ethanol or FAD or NADPH generating system were run simultaneously. Then reaction volume was extracted with 500 μl toluene, the organic phase separated and evaporated under $N_2$ and then silylated with a mixture of BSTFA/acetonitrile (1:2), at 60°C for 15 min. Chromatographic conditions were as follows: column, 5% phenylmethyl silicone, 12 m × 0.2 mm i.d., programmed from 100 to 300°C at a ramp of 10°C/min. Injection port was at 250°C and transfer line to MS, 300°C. Spectra were taken at 70 eV scanning quadrupole from 50 to 550 amu.

### *2.7. Biotransformation of ethanol to 1-hydroxyethyl radical by human cytochrome P450 reductase*

The incubation mixture contained human P450 reductase (0.1 mg prot./ml), 9.4 mM PBN, 0.28 M ethanol and NADPH generating system in 300 mM $K_2HPO_4$ buffer pH 7.4. Control samples without P450 Red or ethanol or NADPH generating system were run simultaneously. In one experiment ICDh was used and P450 Red omitted. After incubation for 1 h at 37° C under $N_2$, the reaction volume (3 ml) was extracted with 500 μl toluene, centrifuged and the organic layer evaporated under nitrogen. The residue was silylated with BSTFA and analyzed by GC-MS in the selected ion monitoring (SIM) mode, to increase sensitivity. Selected masses were 250 (M — $^{\bullet}CHCH_3OTMS$) and 194 ($m/z$ 250 — $C_4H_8$).

### *2.8. Biotransformation of ethanol to 1-hydroxyethyl radical by rat liver microsomes or rat liver nuclei*

Incubation mixture and conditions were as above except that rat liver microsomes (3.5–4 mg prot./ml) or rat liver nuclei (4.5–5 mg prot./ml) were used instead P450 Red. The determination of 1-hydroxyethyl radical was carried out as already described in this section.

### *2.9. Inhibition of rat liver microsomal or nuclear ethanol metabolism to 1-hydroxyethyl radical with antibody against rat liver microsomal NADPH P450 reductase*

Incubation mixture and conditions were as above except that rat liver microsomes (3.5–4 mg

prot./ml) or rat liver nuclei (4.5–5 mg prot./ml) were used instead P450 Red. Determination of 1-hydroxyethyl radical as already described.

### 2.10. Statistics

The significance of the difference between two mean values was assessed by the Student's *t*-test (Graph Pad Software, 1993).

## 3. Results

### 3.1. Biotransformation of ethanol to acetaldehyde under different experimental conditions

In order to see whether pure P450 Red was able to biotransform ethanol to acetaldehyde, incubation mixtures were conducted under air and nitrogen atmospheres. P450 Red was able to biotransform ethanol under air, as already known (Ohnishi and Lieber, 1978; Winston and Cederbaum, 1983, 1986), but it was also able to produce acetaldehyde under an oxygen-free nitrogen atmosphere in a process that required NADPH as cofactor (Table 1). When FAD was included in the incubation mixture, the production of acetaldehyde was significantly enhanced and it can be seen that the reaction is able to proceed anaerobically in the presence of FAD and NADPH, even in the absence of P450 Red (Table 1). On the other hand, the reaction was not able to occur to a significant extent when using ICDh instead of P450 Red in the presence of $NADP^+$ or $NAD^+$ (Table 1).

### 3.2. Inhibition of liver nuclear and microsomal ethanol biotransformation to acetaldehyde with antibody against rat liver microsomal P450 reductase

To check whether anti-rat P450 Red is able to inhibit ethanol biotransformation in hepatic nuclear or microsomal fractions, those subcellular fractions were incubated with the P450 Red antiserum in the presence of NADPH. As shown in Table 2, the antibody against P450 Red was able to inhibit to a significant degree the transformation of ethanol to acetaldehyde in both subcellular fractions, with respect to controls, in which normal serum was used instead of antiserum.

### 3.3. 1-Hydroxyethyl radicals generated by FAD

The GC analysis with TIC detection of reaction products arising when free radicals were generated from ethanol, FAD and NADPH is depicted in Fig. 1. The reduced FAD cofactor was extremely efficient generating 1-hydroxyethyl radicals (peak PBN-1HEt). Molecular mass and fragmentation pattern are in agreement with the adduct reported previously by us in the case of 1HEt radicals generated in a Fenton reaction system (Castro et

Table 1
Biotransformation of ethanol to acetaldehyde by human P450 reductase (P450 Red) and under other experimental conditions

| Experimental condition[a] | Acetaldehyde (ng)/mg prot. |
|---|---|
| NADPH gen. system ($N_2$) | $70.2 \pm 9.7$ |
| P450 Red-NADPH ($N_2$) | $87.9 \pm 6.3$[c] |
| P450 Red + NADPH gen. system ($N_2$) | $818.4 \pm 40.4$[b] |
| P450 Red + NADPH gen. system (air) | $1819.5 \pm 221.4$[b] |
| P450 Red + FAD + NADPH gen. system ($N_2$) | $6379.5 \pm 776.7$[b] |
| FAD + NADPH gen. system ($N_2$) | $4592.4 \pm 272.9$[b] |
| P450 Red + NADPH (1.3 mM) ($N_2$) | $2759.4 \pm 744.6$[b] |
| ICDh + 1.3 mM NADPH ($N_2$) | $87.76 \pm 15.2$[c] |
| ICDh + 0.3 mM NADH ($N_2$) | $86.73 \pm 6.46$[c] |

[a] A 3-ml reaction mixture containing P450 reductase (P450 Red) (0.1 mg prot./ml) when indicated, NADPH generating system (gen. system) when indicated and 0.28 M ethanol in 300 mM $K_2HPO_4$ buffer pH 7.4, was incubated for 1 h at 37°C (under a $N_2$ atmosphere, except in one case). In two experiments P450 Red was replaced by ICDh, in another two, NADPH generating system was replaced by NADPH (1.3 mM) or NAD (0.3 mM) and when indicated FAD 1 mM was used. In one experiment FAD (1 mM + NADPH gen. system) without P 450 Red was used. Acetaldehyde was measured in the head space of each sample after adding 1 ml NaCl saturated solution. Each result is the mean of three determinations. ICDh, isocitric acid dehydrogenase.

[b] $P < 0.001$ with respect to P450 Red–NADPH ($N_2$).

[c] $P > 0.5$ with respect to P450 Red–NADPH ($N_2$).

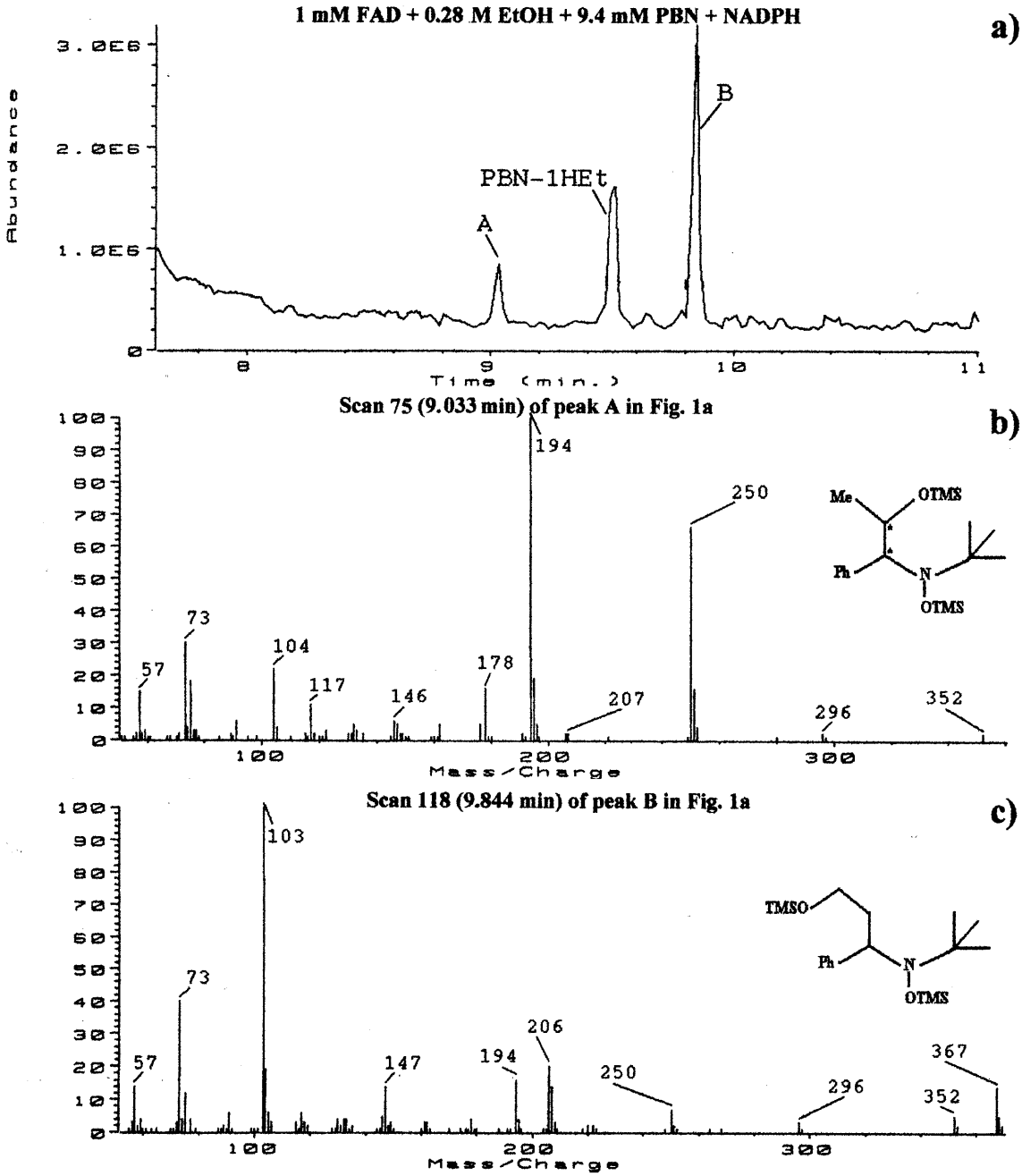



Fig. 1. (a) Gas chromatogram obtained from a sample of the reaction mixture containing PBN and ethanol in the FAD-NADPH model system, after trimethylsilylation. Column, fused silica capillary crosslinked with 5% phenylmethyl silicone, programmed at 10°C/min, from 100 to 300°C. Peaks: A, 1-hydroxyethyl adduct of PBN; PBN-1HEt, diastereomer of A; B, 2-hydroxyethyl adduct of PBN. (b) Mass spectrum taken from peak A in (a). $M^{+\bullet}$ was not present but it was deduced from $m/z$ 352 (M-15), $m/z$ 250 (loss of $^{\bullet}CHCH_3OTMS$) and confirmed by other ions from a fragmentation pattern, typical of many PBN adducts: $m/z$ 296 (loss of isobutylene from M-15), $m/z$ 194 (base peak corresponding to the loss of isobutylene from $m/z$ 250) and $m/z$ 104 (loss of TMSOH from $m/z$ 194). The presence of the 1-hydroxyethyl radical was confirmed by the fragment at $m/z$ 117 ($CH_3CH{=}O^+$-TMS). Chiral carbon centers were denoted by asterisks. (c) Mass spectrum taken from peak B in (a). $M^{+\bullet}$ was observed at $m/z$ 367 and confirmed by fragments at $m/z$ 352 (M-15) and $m/z$ 296 (loss of isobutylene from M-15). Base peak at $m/z$ 103 denoted the presence of a 2-hydroxyethyl moiety attached to the PBN structure ($CH_2O^+$TMS). It was shifted to $m/z$ 105 when ethanol-$d_5$ was used in incubations.

Table 2
Inhibition of rat liver nuclear and microsomal ethanol metabolism to acetaldehyde with antibody against rat P450 reductase

| Subcellular fraction[a] | Acetaldehyde (ng) $\times 10^{-1}$/mg prot. |
|---|---|
| Nuclei (control) | $25.1 \pm 0.7$ |
| Nuclei (antibody) | $16.7 \pm 0.1$[b] |
| Microsomes (control) | $11\,680 \pm 600$ |
| Microsomes (antibody) | $6640 \pm 340$[b] |

[a] Incubation mixtures contained: liver microsomes (40 μg prot./ml) or liver nuclei (4.5–5 mg prot./ml), antibody against rat P450 Red (100 μl), NADPH generating system, 0.28 M ethanol in 300 mM $K_2HPO_4$ buffer, pH 7.4, for the case of microsomes or 0.25 M sucrose in TKM buffer, pH 7.4 for the case of nuclei. Rat antibody was preincubated with microsomes or nuclei at room temperature for 30 min. A control reaction was run using normal serum. Acetaldehyde was measured as described in Section 2.

[b] $P < 0.001$.

al., 1997, 1998). In addition, two more peaks related to ethanol-derived radicals, were detected here. Peak A in Fig. 1 showed a spectrum with a fragmentation pattern similar to that of PBN-1HEt. It was assigned to an isomer (diastereomer) of PBN-1HEt for its structure has two chiral carbon centers. Peak B also presented the same molecular mass but a different fragmentation pattern (Fig. 1b). The base peak at $m/z$ 103 would indicate the presence of a hydroxymethyl group on the molecule ($CH_2O^+TMS$). Replacement of the alcohol by the deuterated analog $d_5$-ethanol in the incubation medium led to a spectrum with the expected mass shifts. Therefore, the peak was assigned to the adduct formed between 2-hydroxyethyl radical and the nitrone.

### *3.4. 1-Hydroxyethyl radicals generated in different in vitro biological systems*

#### *3.4.1. P450 reductase*

The pure enzyme was able to generate 1-hydroxyethyl radicals from ethanol under an anaerobic atmosphere (Fig. 2a). The only peak detected was the one corresponding to the adduct previously observed to be formed in biological samples (Castro et al., 1997, 1998). Its formation was shown to be strongly dependent on the presence of NADPH (Fig. 2b).

#### *3.4.2. ICDh*

No 1HEt formation was observed when pure ICDh was incubated with ethanol in the presence of isocitrate and $NADP^+$ or $NAD^+$.

#### *3.4.3. Liver microsomes*

Results for 1HEt radical generation in microsomal incubations under nitrogen are depicted in Fig. 3a,b. No PBN-1HEt adduct was detected in the absence of NADPH and P450 Red antibody was able to inhibit all the microsomal biotransformation under those experimental conditions (oxygen-free atmosphere).

#### *3.4.4. Liver nuclei*

1HEt radicals were generated by nuclear biotransformation of ethanol to a lower extent than in the case of microsomes but still dependent on the presence of NADPH (Fig. 3c). The reaction was completely inhibited by adding P450 antibody to the incubation mixture (Fig. 3d).

## 4. Discussion

In agreement with the hypothesis that P450 reductase might bioactivate ethanol to acetaldehyde under strictly anaerobic conditions in a process requiring NADPH (Castro et al., 1998), the present experiments using the pure enzyme as the only component in oxygen-free incubation mixtures fully confirmed that possibility. No acetaldehyde formation was observed in the absence of NADPH. The simultaneous formation of 1HEt radicals in the process suggests that the latter is involved in the production of acetaldehyde and also that the bioactivation of ethanol to the 1HEt might involve the flavosemiquinone state of the enzyme. In effect, NADPH-P450 reductase is a flavoprotein containing 1 mol each of FAD and FMN (FAD-E-FMN) (Iyanagni and Mason, 1973; Williams, 1976). Electron transfer proceeds from NADPH to FAD to FMN (Opsian and Coon, 1982; Strobel et al., 1995). This process was thoroughly studied and involves the existence of

several unpaired electron-containing semiquinone forms e.g. [FADH$^{\bullet}$-E-FMN], [FAD-E-FMNH$^{\bullet}$]; [FADH$^{\bullet}$-E-FMNH$^{\bullet}$], [FADH$^{\bullet}$-E-FMH$_2$] and [FADH$_2$-E-FMNH$^{\bullet}$] (Opsian and Coon, 1982; Müller, 1987). These semiquinone forms might be involved in the generation of 1HEt from EtOH. That possibility is further suggested by our studies using pure FAD, which are useful to gain an insight into the chemical behavior of the flavin moiety. In those studies we found that reduced FAD is able to generate 1HEt. Under this proposed mechanism, acetaldehyde would arise from a rearrangement of the 1HEt by splitting of a hydrogen atom. We observed that the reducing agent might be either NADPH or NADH. However under biological conditions NADPH would be the preferred source of reducing equivalent to P450 reductase (reviews in Jäning and Pfeil, 1984; Strobel et al., 1995). The interaction of ethanol with the enzyme when it is in its proper membrane site might be at its hydrophilic domain protruding the membrane surface rather than at the hydrophobic one holding the molecule in the membrane. This possibility is more likely than the opposite in view of the hydrophilic nature of ethanol. In addition that site also contains the binding site for the cosubstrate transferring the electron to the flavins (Jäning and Pfeil, 1984; Strobel et al., 1995). Our studies using specific antibody evidenced that these P450 reductase-mediated pathways of ethanol biotransformation to acetaldehyde and 1HEt may also operate when the enzyme is either in the microsomal or in the nuclear membrane (Philips and Langdon, 1962; Williams and Kamin, 1962; Kasper, 1971).

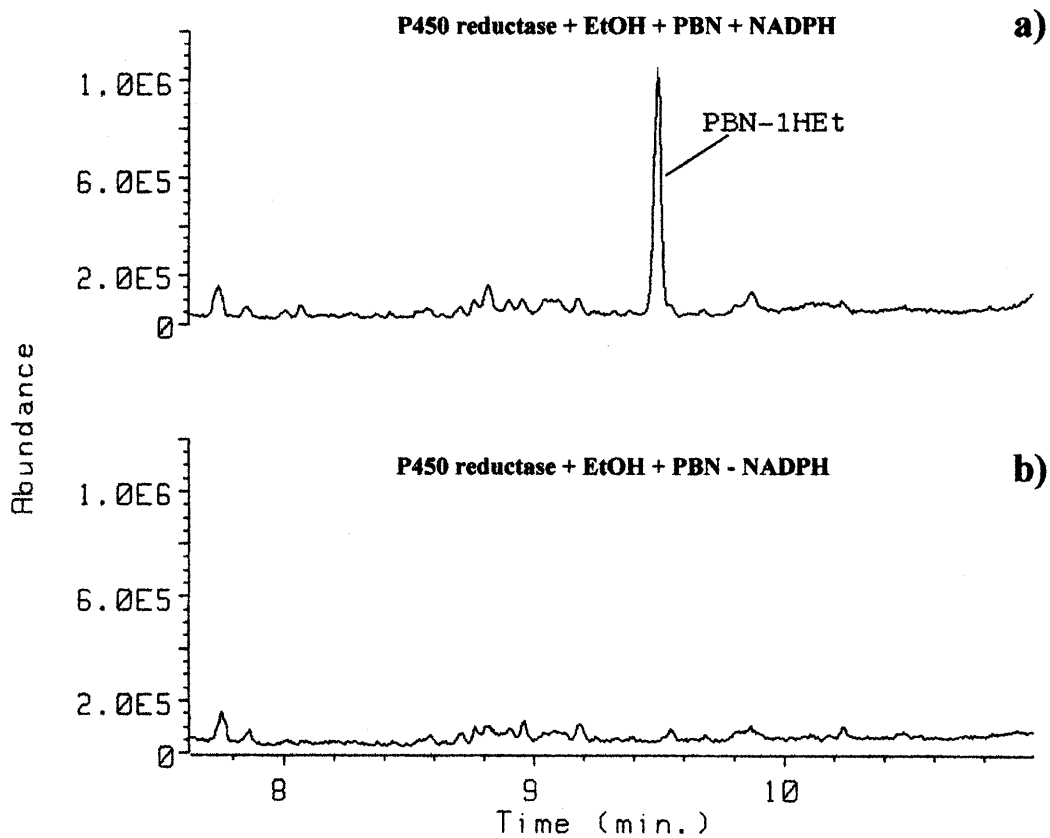
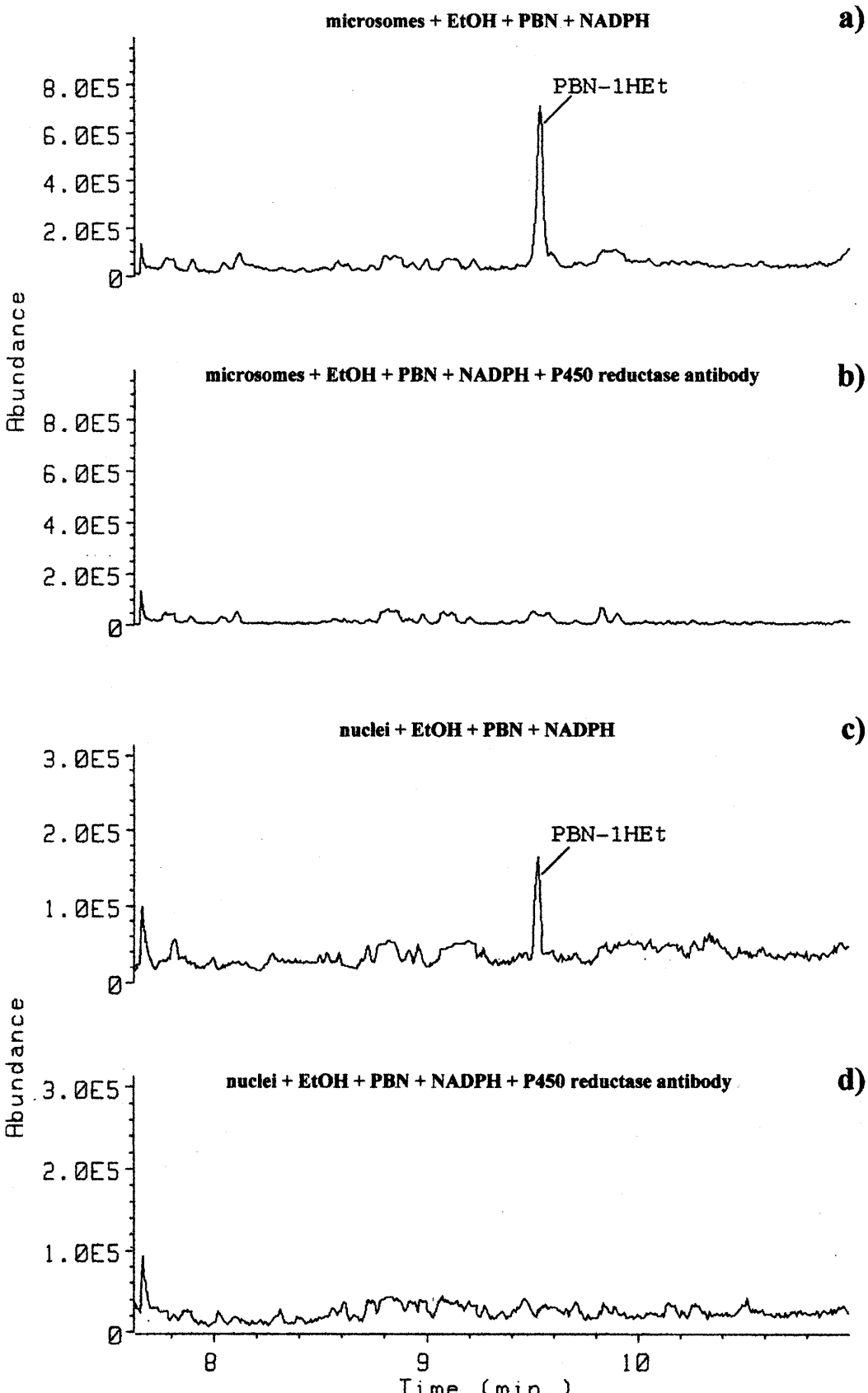



Fig. 2. (a) Selected-ion current profile obtained from GC-MS-SIM analysis of a sample of incubation containing pure P450 reductase, NADPH, ethanol and PBN, under nitrogen atmosphere and after trimethylsilylation. Masses selected for SIM were 250 and 194. See Section 2 for details. (b) The same as in (a) but in the absence of NADPH.


microsomes + EtOH + PBN + NADPH
a)
PBN-1HEt
8.0E5
6.0E5
4.0E5
2.0E5
0
Abundance
microsomes + EtOH + PBN + NADPH + P450 reductase antibody
b)
nuclei + EtOH + PBN + NADPH
c)
3.0E5
2.0E5
1.0E5
nuclei + EtOH + PBN + NADPH + P450 reductase antibody
d)
8
9
10
Time (min.)

In the presence of oxygen, P450 reductase may also directly oxidize ethanol via a different mechanism involving the formation of oxygen reactive species and both in the absence or in the presence of adequate cytochrome P450 isoforms (e.g. CYP2E1) (Ohnishi and Lieber, 1978; Winston and Cederbaum, 1983, 1986). In tissues where those P450 isoforms are not present or they are in non-significant proportions, a direct P450 reductase mediated pathway may be operating. In this context it may be worth considering that P450 reductase has no known isoforms (Jäning and Pfeil, 1984; Strobel et al., 1995) and that it was found to be present not only in the liver endoplasmic reticulum but also in the nuclear membrane (Kasper, 1971) and that it was evidenced to be widely distributed throughout the constituent tissues of most mammals and even other species (Villarruel et al., 1977; Jäning and Pfeil, 1984). A potential additional implication of the present observations might be that other flavoproteins of the dehydrogenase or reductase classes were able to activate ethanol to acetaldehyde and 1HEt. In effect, this class of flavoproteins utilize the flavosemiquinone state and react rapidly with electron acceptors, while the other two classes of flavoenzymes (e.g. oxidases and monooxygenases) do not (Müller, 1987). That possibility, however, remains to be confirmed.

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Fig. 3. (a) Selected-ion current profile obtained from GC-MS-SIM analysis of a sample of incubation containing microsomes, NADPH, ethanol and PBN, under nitrogen atmosphere and after trimethylsilylation. Masses selected for SIM were 250 and 194. See Section 2 for details. (b) The same as in (a) but in the presence of P450 reductase antibody. (c) Selected-ion current profile obtained from GC-MS-SIM analysis of a sample of incubation containing purified nuclei, NADPH, ethanol and PBN, under nitrogen atmosphere and after trimethylsilylation. (d) The same as in (c) but in the presence of P450 reductase antibody.

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