Effect of oxygen concentration on microsomal oxidation of ethanol and generation of oxygen radicals

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The iron-catalysed production of hydroxyl radicals, by rat liver microsomes (microsomal fractions), assessed by the oxidation of substrate scavengers and ethanol, displayed a biphasic response to the concentration of O_2 (varied from 3 to 70 %), reaching a maximal value with 20 % O_2 . The decreased rates of hydroxyl-radical generation at lower O_2 concentrations correlates with lower rates of production of H_2O_2 , the precursor of hydroxyl radical, whereas the decreased rates at elevated O_2 concentrations correlate with lower rates (relative to 20 % O_2) of activity of NADPH-cytochrome *P*-450 reductase, which reduces iron and is responsible for redox cycling of iron by the microsomes. The oxidation of aniline or aminopyrine and the cytochrome *P*-450/oxygen-radical-independent oxidation of ethanol also displayed a biphasic response to the concentration of O_2 , reaching a maximum at 20 % O_2 , which correlates with the dithionite-reducible CO-binding spectra of cytochrome *P*-450. Microsomal lipid peroxidation increased as the concentration of O_2 was raised from 3 to 7 to 20 % O_2 , and then began to level off. This different pattern of malondialdehyde generation compared with hydroxyl-radical production probably reflects the lack of a role for hydroxyl radical in microsomal lipid peroxidation. These results point to the complex role for O_2 in microsomal generation of oxygen radicals, which is due in part to the critical necessity for maintaining the redox state of autoxidizable components of the reaction system.

INTRODUCTION

Microsomes (microsomal fractions) generate superoxide ($O_2^{\cdot-}$) and H_2O_2 during NADPH-dependent electron transport, with NADPH-cytochrome *P*-450 reductase and cytochrome *P*-450 being the major loci for the production of various oxidants (Sato, 1967; Strobel & Coon, 1971; Aust *et al.*, 1972; Prough & Masters, 1973; Dybing *et al.*, 1976; Werringloer *et al.*, 1977). In the presence of certain iron complexes, potent oxidants such as the hydroxyl radical ($\cdot OH$) are produced (Fong *et al.*, 1973; Aust & Svingen, 1982; Winston *et al.*, 1984; Aust *et al.*, 1985). The present study concerns an evaluation of the effect of varying the concentration of O_2 on microsomal generation of $\cdot OH$ and other oxidants. It would be expected that increasing the concentration of O_2 should promote the production of various oxidants by microsomes, since, during hyperoxia, cellular $O_2^{\cdot-}$ and H_2O_2 are increased (Gerschman, 1964; Chance *et al.*, 1979; Freeman & Crapo, 1981; Turrens *et al.*, 1982*a,b*; Jones, 1985), and induction of the Mn and the Cu–Zn superoxide dismutase occurs (Crapo & Tierney, 1974). In studies with lung microsomes and mitochondria, increasing the concentration of O_2 from 21 to 100 % resulted in nearly a 2-fold increase in H_2O_2 production (Turrens *et al.*, 1982*a*). On the other hand, the critical role of hypoxia in producing cellular damage via lipid peroxidation produced by redox-cycling agents has also been emphasized (Kappus & Sies, 1981; Borg & Schaich, 1984; Jones, 1985), as agents such as halothane or carbon tetrachloride have been found to produce lipid peroxidation and hepatotoxicity within certain narrow limits of O_2 concentration (DeGroot & Noll, 1984, 1986).

Since the rate of oxygen-radical production depends on the O_2 partial pressure and the redox state of autoxidizable redox components such as reduced reductase, reduced cytochrome *P*-450, ferrous or other agents capable of redox cycling, experiments were carried out with rat liver microsomes incubated with hypoxic, normoxic and hyperoxic concentrations of O_2 , and the effects on microsomal generation of H_2O_2 and $\cdot OH$ and on lipid peroxidation were evaluated. In addition, certain drugs such as ethanol, which are primarily oxidized by a cytochrome *P*-450-dependent process independent of $\cdot OH$ (Koop *et al.*, 1982; Winston *et al.*, 1983), can become oxidized by a $\cdot OH$ -dependent process under conditions in which microsomal generation of oxygen radicals is increased, e.g. in the presence of redox-cycling agents and iron complexes (Feierman & Cederbaum, 1983; Winston & Cederbaum, 1983*b*; Belouqui & Cederbaum, 1985). Therefore the oxidation of ethanol by $\cdot OH$ -dependent and independent microsomal reactions was also assessed in the presence of different concentrations of O_2 , and results were compared with those found for other drug substrates or $\cdot OH$ -scavenging agents. The overall aim of the study was to evaluate the role of the concentration of oxygen on microsomal generation of oxygen radicals and on ethanol oxidation.

MATERIALS AND METHODS

Liver microsomes were prepared from male Sprague–Dawley rats weighing about 150–200 g. The microsomes were prepared by differential centrifugation, washed twice with 125 mM-KCl and stored at $-70^\circ C$ until utilized. The content of cytochrome *P*-450 was

Abbreviations used: KMB, 2-oxo-4-thiomethylbutyrate; DMSO, dimethyl sulphoxide.

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determined by the method of Omura & Sato (1964), and the activity of NADPH-cytochrome *P*-450 (cytochrome *c*) reductase was determined by the method of Phillips & Langdon (1962), in 300 mM-phosphate buffer, pH 7.4. The phosphate buffer and the water utilized to prepare all solutions were passed through columns of Chelex-100 resin to remove metals such as iron.

Ethanol oxidation was determined at 37 °C in a basic reaction system consisting of 100 mM-potassium phosphate, pH 7.4, 10 mM-MgCl₂, 0.4 mM-NADP⁺, 1 mM-NaN₃, 50 mM-ethanol and about 2 mg of microsomal protein in a final volume of 1.0 ml. The reaction was initiated by the addition of glucose 6-phosphate (final concn. 10 mM) plus 1 unit of glucose-6-phosphate dehydrogenase and terminated after 5 min by addition of 0.3 ml of 1 M-HCl. In some experiments, 50 μM-Fe³⁺-100 μM-EDTA was added to catalyse the production of [•]OH by the microsomes (Winston *et al.*, 1984). Before addition of the microsomes, the flasks plus other reaction contents were gassed for 15 s with gas mixtures containing 3% O₂+97% N₂, 7% O₂+93% N₂, 20% O₂+80% N₂, 47% O₂+53% N₂, or 70% O₂+30% N₂. Gas mixtures were obtained from T. W. Smith, Specialty Gas Product Group (Union Carbide Corp., Danbury, CT, U.S.A.). The microsomes were added and the flasks again gassed with the appropriate mixture for 30 s before being sealed with a rubber serum cap. Reactions were initiated and terminated by injecting the generating system or the HCl through the serum cap with a syringe. This gassing procedure was followed for all the other reactions described below. The production of acetaldehyde was determined by a headspace g.l.c. procedure (Cederbaum & Cohen, 1984). All values were corrected for zero-time controls, in which the HCl was injected before the generating system.

The oxidation of aminopyrine or aniline was determined with the same basic incubation medium as for ethanol, except that 5 mM-aminopyrine or 1 mM-aniline replaced the ethanol, NaN₃ was omitted, and microsomal protein was about 1 mg per flask. Reactions were conducted for 5–10 min and were terminated by the addition of 0.3 ml of 20% (w/v) trichloroacetic acid. The production of formaldehyde from aminopyrine was assayed by the Nash (1953) reaction, and the production of *p*-aminophenol from aniline was determined by the method of Cochin & Axelrod (1959).

The production of [•]OH or compounds with the oxidizing power of [•]OH by microsomes was assayed by measuring the generation of ethylene gas from 2-oxo-4-thiomethylbutyrate (KMB) or of formaldehyde from dimethyl sulphoxide (DMSO) (Cederbaum & Cohen, 1984, 1985). The basic reaction system was identical with that for ethanol oxidation, except for the use of either 10 mM-KMB or 33 mM-DMSO in place of the ethanol. Reactions were carried out in the absence or presence of 50 μM-Fe³⁺-100 μM-EDTA, for either 5–15 min (KMB) or 10–35 min (DMSO) before termination with either HCl (KMB) or trichloroacetic acid (DMSO). The production of formaldehyde was determined by the Nash (1953) reaction, and the generation of ethylene was assayed by headspace g.l.c. All values were corrected for zero-time controls.

Microsomal lipid peroxidation was assayed by the production of thiobarbituric acid-reactive components. Reactions were carried out in a system containing 60 mM-Tris buffer, pH 7.4, 10 mM-MgCl₂, 50 μM-ferric

ammonium sulphate, the NADPH-generating system and about 1 mg of microsomal protein in a final volume of 1 ml. Reactions were carried out for 2 or 6 min and terminated by the addition of 0.3 ml of 20% trichloroacetic acid. Malondialdehyde was determined by the thiobarbituric acid reaction (Buege & Aust, 1978). The production of H₂O₂ was determined by measuring the generation of formaldehyde from the oxidation of methanol by the catalase-compound I complex (Hildebrandt *et al.*, 1978). Reactions were carried out in 200 mM-KCl/50 mM-Tris, pH 7.4, containing 10 mM-MgCl₂, 100 mM-methanol, 300 units of catalase, the NADPH-generating system and about 3 mg of microsomal protein in a final volume of 2 ml. Reactions were terminated after 5 min by addition of 1 ml of 20% trichloroacetic acid, and the formaldehyde produced was determined by the Nash (1953) reaction.

The butan-1-ol binding spectrum was obtained with a Perkin-Elmer model 554 dual-beam spectrophotometer. Microsomes were incubated at 37 °C in 100 mM-phosphate (pH 7.4)/10 mM-MgCl₂ buffer under different concentrations of O₂. After incubation for 10 min, the flasks were opened, and the microsomal mixture was diluted 10-fold by addition of 100 mM-phosphate. The samples were divided into two cuvettes, and, after a baseline correction, 30 mM-butan-1-ol was added to the sample cuvette and the spectrum scanned over the range 500–350 nm.

The model ascorbate reaction system consisted of 100 mM-phosphate/10 mM-MgCl₂, pH 7.4, and either 10 mM-KMB or 50 mM-ethanol in a final volume of 1.0 ml. Reactions were carried out in the absence or presence of 50 μM-Fe³⁺-100 μM-EDTA and were initiated by the addition of ascorbic acid (final concn. 2 mM). Reactions were quenched after 5 or 10 min by addition of 0.5 M-HCl, and the production of acetaldehyde or ethylene was determined by headspace g.l.c. The production of H₂O₂ by the ascorbate system was determined essentially as described for the microsomal system, where all values refer to means ± S.E.M.

RESULTS

Microsomal oxidation of ethanol

The oxidation of ethanol by rat liver microsomes proceeds primarily by a cytochrome *P*-450-dependent reaction (Cederbaum, 1987; Winston & Cederbaum, 1983a,b). When Fe³⁺-EDTA is added, microsomes generate considerable amounts of [•]OH, and a second reaction for ethanol oxidation, the [•]OH-dependent oxidation of ethanol, becomes significant (Feierman & Cederbaum, 1983; Winston *et al.*, 1984). Differences in rates of ethanol oxidation in the presence and the absence of Fe³⁺-EDTA reflect the [•]OH-dependent rate of ethanol oxidation. A biphasic response to the concentration of O₂ was observed for the cytochrome *P*-450-dependent rate of oxidation of ethanol (without Fe³⁺-EDTA), total rates of ethanol oxidation (with Fe³⁺-EDTA), and for the [•]OH-dependent rate of oxidation of ethanol (Table 1). Maximal activity was observed in the presence of 20% O₂ for all reaction conditions, and activity decreased as the concentration of O₂ was either elevated or decreased (Table 1).

Alcohols interact with cytochrome *P*-450 to produce reversed type I binding spectra (Rubin *et al.*, 1971). Fig. 1 shows a binding spectrum with 30 mM-butan-1-ol; a

Table 1. Effect of varying the O₂ concentration on microsomal oxidation of ethanol

NADPH-dependent microsomal oxidation of ethanol was assayed as described in the Materials and methods section in the absence or presence of 50 μM -Fe³⁺-100 μM -EDTA. The ' $\Delta\text{Fe-EDTA}$ ' column refers to rates in the presence of Fe³⁺-EDTA minus rates in its absence and reflects the 'OH-dependent oxidation of ethanol. Numbers in parentheses are percentages of the values with 20% O₂. Results are means $\pm$ S.E.M. from three experiments.

Concn. of O ₂ (%)	Rate of ethanol oxidation (nmol/min per mg of protein)		
	-Fe-EDTA	+Fe-EDTA	$\Delta\text{Fe-EDTA}$
3	2.16 $\pm$ 0.18 (57)	7.49 $\pm$ 0.37 (51)	5.33 $\pm$ 0.28 (49)
7	2.64 $\pm$ 0.38 (70)	11.84 $\pm$ 0.36 (81)	9.20 $\pm$ 0.37 (84)
20	3.76 $\pm$ 0.04 (100)	14.57 $\pm$ 0.20 (100)	10.81 $\pm$ 0.12 (100)
47	1.86 $\pm$ 0.24 (49)	10.94 $\pm$ 0.60 (75)	9.08 $\pm$ 0.42 (83)
70	1.80 $\pm$ 0.04 (48)	8.52 $\pm$ 0.07 (58)	6.72 $\pm$ 0.05 (62)

Fig. 1. Binding spectrum for the interaction of butan-1-ol with microsomes

Microsomes were incubated for 10 min at 37 °C with 3, 7, 20, 47 and 70% O₂ (panels *a*, *b*, *d*, *e* and *f* respectively); the flasks were then opened, butan-1-ol was added to a final concentration of 30 mM, and the spectrum was scanned over the wavelength range 500–350 nm. In panel (*c*), the microsomes were left on ice and exposed to air before determination of the butan-1-ol-binding spectrum. In each spectrum, the top arrow defines the peak at 418 nm and the bottom arrow shows the beginning of the trough at 390 nm.

peak is observed at about 418 nm and a trough at about 385–390 nm. In Fig. 1(*d*), the microsomes were incubated under air for 10 min at 37 °C and then immediately transferred to a cuvette, and the spectrum was determined on addition of butan-1-ol. In Figs. 1(*a*), 1(*b*), 1(*e*) and 1(*f*) respectively, the microsomes were incubated with 3%, 7%, 47% and 70% O₂ for 10 min at 37 °C before addition of butan-1-ol and determination of the spectrum. Analogous to the results on ethanol oxidation, a biphasic response of the butan-1-ol binding spectrum to varying O₂ concentrations was observed, with the maximal and most distinct binding spectrum being found at 20% O₂. The ΔA of the peak-to-trough absorbance difference (A_{418} minus $A_{385-390}$) was decreased about 25, 20, 25 and 32% at O₂ concentrations of 3, 7, 47 and 70% respectively, compared with the values with 20% O₂. Fig. 1(*c*) depicts a spectrum of microsomes kept on ice (under air) until addition of butan-1-ol, followed immediately by determination of the binding spectrum. Comparisons of Figs. 1(*c*) and 1(*d*) indicate that, even when microsomes are incubated under air, there is some decrease (about 20% of the peak-to-trough ΔA) in the binding spectrum as compared with non-incubated samples.

Microsomal oxidation of drugs

To evaluate the specificity of this effect of O₂ concentration, the oxidation of other typical substrates for cytochrome *P*-450 was determined. Fig. 2 compares results with ethanol with those obtained with either aniline or aminopyrine as substrate. The response of the drug-oxidation curve to varying O₂ concentrations was identical for all three substrates.

Oxidation of 'OH-scavenging agents

To evaluate in a more direct manner the effect of varying the O₂ concentration on microsomal generation of 'OH, scavengers of 'OH which are not effective substrates for cytochrome *P*-450 were utilized. The production of ethylene from KMB, although not specific for 'OH (Pryor & Tang, 1978; Winston *et al.*, 1983), is a sensitive assay in this regard. Microsomes oxidize KMB to ethylene in a reaction which is strikingly increased by the addition of Fe³⁺-EDTA (Winston *et al.*, 1984). When the concentration of O₂ was varied, maximal rates of ethylene production from KMB were found with 20%

Fig. 2. Effect of different concentrations of O₂ on microsomal oxidation of aniline (○), aminopyrine (△) or ethanol (●)

Experiments were carried out as described in the Materials and methods section. Ethanol oxidation was assayed in the absence of Fe³⁺-EDTA and therefore reflects the cytochrome *P*-450-dependent rate. Results are from three experiments.

Fig. 3. Effect of different concentrations of O₂ on microsomal oxidation of KMB

The production of ethylene from 10 mM-KMB was assayed as described in the Materials and methods section in the absence (○) and the presence (●) of 50 μM-Fe³⁺-100 μM-EDTA. Results are from four experiments.

O₂, in both the absence and the presence of Fe³⁺-EDTA (Fig. 3).

Similar experiments were conducted with DMSO as the 'OH-scavenger substrate. The production of formaldehyde has been shown to be a major product resulting from the interaction of DMSO with 'OH

Fig. 4. Effect of different concentrations of O₂ on microsomal production of H₂O₂

Reactions were carried out as described in the Materials and methods section in the absence (○) or presence (●) of Fe³⁺-EDTA. Results are from three experiments.

generated by microsomes (Klein *et al.*, 1981). Rates of formaldehyde production were (nmol/min per mg of protein) 0.50, 0.63, 0.89, 0.55 and 0.43 when microsomes were incubated with 3, 7, 20, 47 or 70 % O₂ respectively (*n* = 2).

Accumulation of H₂O₂

The oxidation of 'OH-scavenging agents by microsomes is decreased by enzymes such as catalase or glutathione peroxidase (plus glutathione) which decompose H₂O₂ (Cederbaum & Cohen, 1984, 1985; Belouqui & Cederbaum, 1986). This suggests that H₂O₂ is the precursor of the oxidant responsible for the oxidation of the 'OH scavengers. In the absence of Fe³⁺-EDTA, most of the H₂O₂ is likely to result from decay of the oxy- or peroxy-cytochrome *P*-450 complex (Kuthan & Ullrich, 1982; Kuthan *et al.*, 1978). In the presence of Fe³⁺-EDTA, H₂O₂ production is increased, primarily because of reduction by the reductase to the ferrous redox state, followed by autooxidation to produce O₂^{•-}, and subsequently H₂O₂ (see the Discussion section).

As shown in Fig. 4, in the absence of Fe³⁺-EDTA, H₂O₂ accumulated in the medium at an increasing rate as the concentration of O₂ was increased from 3 to 7 to 20%, and then levelled off. In the presence of Fe³⁺-EDTA, H₂O₂ production continued to increase as the concentration of O₂ was increased, with the sharpest increase occurring during the transition from lowest O₂ concentration (3%) to 7% O₂. The levelling off of rates of H₂O₂ production at 20% O₂ (about 225 μmol of O₂/ml) is in agreement with previous findings that rates of H₂O₂ production were maximal at O₂ concentrations of 200–300 μM (Thurman *et al.*, 1972) and that the apparent *K_m* for NADPH oxidase activity for O₂ was about 150 μM (Staudinger & Zubrzycki, 1965).

Microsomal lipid peroxidation

The sensitivity of microsomal lipid peroxidation, as catalysed by ferric ammonium sulphate, to various O₂ concentrations was determined by assaying for thio-barbituric acid-reactive products. As shown in Fig. 5, the pattern found for malondialdehyde production was similar to that found with H₂O₂ production, in that increasing activity was obtained as the concentration of O₂ was elevated from 3 to 20 %, followed by a levelling off at elevated concentrations of O₂. In contrast with results involving [•]OH production, microsomal lipid peroxidation did not exhibit a biphasic response to O₂ concentration.

Ascorbate-dependent oxidation of ethanol and KMB

To evaluate whether the biphasic response of the microsomal [•]OH-dependent oxidation of ethanol or

Fig. 5. Effect of different concentrations of O₂ on microsomal lipid peroxidation as assayed by production of malondialdehyde

Reactions were carried out as described in the Materials and methods section in the presence of 50 μ M-ferric ammonium sulphate. Results are from three experiments.

KMB to different oxygen concentrations reflected the actual generation of [•]OH, or rather subsequent reactions involving the interaction of generated [•]OH with the scavengers to yield products, the effect of varying the oxygen concentration on the oxidations of ethanol or KMB by [•]OH produced by a chemical [•]OH-generating system was determined. For this purpose, the Fe³⁺-EDTA-catalysed autoxidation of ascorbate was utilized. Rates of ethanol or KMB oxidation were determined in the absence and presence of Fe³⁺-EDTA, and the difference was calculated to reflect the [•]OH- or Fe³⁺-EDTA-dependent reaction. As shown in Table 2, in the absence of Fe³⁺-EDTA, rates of ethanol and KMB oxidation were relatively constant as the percentage of oxygen was elevated from 3 to 70 %. In the presence of Fe³⁺-EDTA, rates of ethanol and KMB oxidation increased as the oxygen concentration was elevated. A similar trend was observed for the values obtained by subtracting the rates in the absence of Fe³⁺-EDTA from those in its presence (Δ values in Table 2). Thus, in contrast with results with microsomes, the oxidation of ethanol and KMB by [•]OH generated from a chemical system displayed a continual increase in product formation as the concentration of oxygen was elevated.

H₂O₂ is the precursor of the oxidant generated by the Fe³⁺-EDTA-catalysed autoxidation of ascorbate system. In the absence and the presence of Fe³⁺-EDTA, rates of H₂O₂ generation were elevated as the oxygen concentration was increased (Table 2). It appears that increased generation of H₂O₂ as the oxygen concentration increased plays an important role in the production of [•]OH, and consequently in the enhanced oxidation of ethanol and KMB at elevated O₂ concentrations.

Content of cytochrome P-450 and activity of NADPH-cytochrome P-450 reductase

The effect of varying the O₂ concentration on the dithionite-reduced CO spectrum of cytochrome P-450 was determined after incubation of the microsomes for 10 min at 37 °C in the presence of the indicated concentration of O₂, followed by immediate determination of the binding spectrum. In the absence of NADPH, microsomal cytochrome P-450 content, as assessed by the spectrum, was about 0.9 nmol/mg after

Table 2. Ascorbate-dependent oxidation of ethanol and KMB, and H₂O₂ generation

The oxidation of 50 mM-ethanol or 10 mM-KMB or production of H₂O₂ by the ascorbate chemical system was determined as described in the Materials and methods section in the absence or presence of 50 μ M-Fe³⁺-100 μ M-EDTA. Results are means from two experiments.

Reaction conditions	O ₂ (%) ...	Rate of product formation (nmol/min)				
		3	7	20	47	70
Ethanol oxidation	- Fe-EDTA	41	48	49	35	35
	+ Fe-EDTA	51	63	92	99	108
	Δ Fe-EDTA	10	15	43	64	73
KMB oxidation	- Fe-EDTA	6	9	9	9	14
	+ Fe-EDTA	7	18	24	31	37
	Δ Fe-EDTA	1	9	16	22	23
H ₂ O ₂ production	- Fe-EDTA	10	11	26	36	72
	+ Fe-EDTA	44	75	110	177	185
	Δ Fe-EDTA	34	64	84	141	113

Table 3. Effect of varying the concentration of O₂ on the content of cytochrome *P*-450 and activity of NADPH-cytochrome *P*-450 reductase

Microsomes were incubated for 10 min with the indicated concentration of O₂ at 37 °C in either the absence or the presence of an NADPH-generating system. The flasks were then opened and the cytochrome *P*-450 content (as assessed by the dithionite-reduced CO-binding spectrum) or the activity of the reductase was immediately determined. Numbers in parentheses are percentages of the values found with 20 % O₂. Results are means from two experiments.

Reaction	Concn. of O ₂ (%)	Content or activity	
		– NADPH	+ NADPH
Cytochrome <i>P</i> -450 (nmol/mg of protein)	3	0.56 (61)	0.36 (72)
	7	0.54 (59)	0.37 (74)
	20	0.92 (100)	0.50 (100)
	47	0.54 (59)	0.45 (90)
	70	0.50 (54)	0.31 (62)
NADPH-cytochrome <i>P</i> -450 reductase (nmol/min per mg of protein)	3	168 (100)	113 (79)
	7	163 (97)	138 (97)
	20	168 (100)	143 (100)
	47	133 (79)	83 (58)
	70	99 (59)	37 (26)

10 min incubation in 20 % O₂. Incubation in the absence of NADPH with either lower or higher concentrations of O₂ caused a decrease in the cytochrome *P*-450 binding spectrum (Table 3). When the NADPH-generating system was included in the reaction system, the content of 'active' cytochrome *P*-450 was lower at all O₂ concentrations after 10 min incubation as compared with values obtained in the absence of NADPH; this was especially notable for samples incubated with air, which had the highest control values of total cytochrome *P*-450 to begin with (Table 3). The response of the binding spectrum of cytochrome *P*-450 to different O₂ con-

of studies concerning halothane-induced lipid peroxidation and hepatotoxicity, in which little or no lipid peroxidation was observed at O₂ concentrations below 1 mmHg or above 10 mmHg (DeGroot & Noll, 1984, 1986). In these studies with halothane, it was suggested that the *p*O₂ must be low enough to permit reductive formation of the halothane radical, but high enough for lipid peroxidation to occur.

Previous experiments have established the following mechanism for the generation of [•]OH by the microsomes in the presence of an iron catalyst such as Fe³⁺-EDTA:

centrations was essentially similar in the absence and the presence of NADPH, namely, the maximal spectrum was found with 20 % O₂, with decreasing values occurring as the concentration of O₂ was either elevated or lowered (Table 3).

Similar experiments were carried out to evaluate the effect of O₂ concentration on the activity of NADPH-cytochrome *P*-450 reductase. In the absence of NADPH (during the incubation), the activity of the reductase was the same for microsomes incubated with 3, 7 and 20 % O₂, and then activity decreased when the microsomes were incubated with higher concentrations of O₂ (Table 3). Similar results were obtained when NADPH was included in the incubation system; the decrease in activity of the reductase at the higher concentrations of O₂ was, however, greater when NADPH was present during the incubation period (Table 3).

DISCUSSION

The oxidation of [•]OH scavengers, drugs and ethanol by microsomes displays a biphasic response to the concentration of O₂. This biphasic response is reminiscent

(Winston & Cederbaum, 1983*a,b*; Krikun & Cederbaum, 1985; Belouqui & Cederbaum, 1986). At low concentrations of O₂ (3 or 7 %), the generation of H₂O₂ by microsomes is smaller as compared with values found with 20 % O₂ (Fig. 4). Since H₂O₂ is the precursor of [•]OH (reaction 4), the lower rates of H₂O₂ generation are probably a major factor in the decreased rates of [•]OH generation found at lower O₂ concentrations. Similar results are observed with the chemical ascorbate system. However, in contrast with results with the ascorbate system, increasing the concentration of O₂ to values greater than 20 % results in a decrease in [•]OH generation by microsomes. This decrease does not reflect rates of H₂O₂ generation, which show a continuous, although less pronounced, increase (presence of Fe³⁺-EDTA) as the concentration of O₂ increases. Indeed, decreased production of [•]OH from H₂O₂ may contribute to the accumulation of H₂O₂ at elevated concentrations of O₂. The activity of NADPH-cytochrome *P*-450 reductase is decreased when microsomes are incubated with 47 and 70 % O₂ as compared with activity at 20 % O₂ (Table 3). Microsomal [•]OH generation is not sensitive to superoxide dismutase, which suggests that reduction and subsequent redox cycling of the iron-chelate occurs primarily via the

reductase (reaction 1). Decreased activity of the reductase as the concentration of O₂ is elevated could be responsible, at least in part, for lower rates of [•]OH generation at the higher O₂ concentrations. A possible mechanism for this decreased activity may involve the fact that the reductase has essential thiol groups, which are critical for activity (Masters *et al.*, 1965; Franklin & Estabrook, 1971). Higher concentrations of O₂ have long been known to be toxic to a variety of thiol-containing enzymes (Balentine, 1982; Halliwell, 1981). Results in Table 3 also show that the activity of the reductase is more sensitive to elevated O₂ concentrations in the presence of an NADPH-generating system, which may reflect the additional stress of various oxidants generated by microsomal electron transfer, e.g. O₂^{•-} or H₂O₂, on enzyme activity. Such considerations would obviously not apply to the ascorbate system.

An additional contributing factor to the lower rates of [•]OH generation at elevated concentrations of O₂ could involve the competition between reactions (2) and (4) for the ferrous complex. This possibility would point to the critical necessity of maintaining appropriate steady-state ferrous concentrations and the effect of O₂ concentrations on the redox state of autoxidizable reaction components (Kappus & Sies, 1981; Borg & Schaich, 1984; Jones, 1985).

The cytochrome *P*-450-dependent oxidation of ethanol, as well as the oxidation of aniline or aminopyrine and the butan-1-ol binding spectrum, displayed a biphasic response to the concentration of O₂. It is unlikely that the amount of O₂ is limiting for mixed-function oxidase activity, since the apparent *K_m* for O₂ of liver microsomes is very low (Imai & Sato, 1968; Kampfmeyer & Kiese, 1964). The drug oxidation response to O₂ concentration is similar to the pattern found for the CO-binding spectrum of cytochrome *P*-450. At all concentrations of O₂, the magnitude of the spectrum of cytochrome *P*-450 was lower when microsomes were incubated in the presence of NADPH; this probably reflects the destruction of cytochrome *P*-450 by H₂O₂ and lipid hydroperoxides formed during microsomal electron transfer (Guengerich, 1978; Levin *et al.*, 1973). The decrease in drug oxidation at elevated concentrations of O₂ may also reflect the decrease in activity of the reductase which occurs in the presence of NADPH and high O₂ concentrations. It is, however, not clear why the magnitude of the spectrum of cytochrome *P*-450 is lower at 3 or 7% O₂ as compared with 20% O₂, since H₂O₂ generation and lipid peroxidation are also lower at 3 and 7% concentrations of O₂.

The effect of O₂ concentration on microsomal lipid peroxidation appears to be complex and depends on the nature of products assayed (Kostrucha & Kappus, 1986; Reiter & Burk, 1987). Results in Fig. 5 confirm several reports that microsomal lipid peroxidation assessed as malondialdehyde production increases as the O₂ concentration is elevated, and then levels off (Kostrucha & Kappus, 1986; Reiter & Burke, 1987; Noll *et al.*, 1987). The levelling off of malondialdehyde production at elevated O₂ concentrations could reflect the decreased activity of the reductase, which is involved in the initiation step of lipid peroxidation, and perhaps the decreased content of cytochrome *P*-450, which may play a role in the degradation of lipid hydroperoxides (Aust *et al.*, 1985; Svingen *et al.*, 1979; Hrycay & O'Brien, 1971). That the pattern of response of malondialdehyde

generation to O₂ concentration differs from that of [•]OH generation probably reflects the fact that free [•]OH does not play a significant role in microsomal lipid peroxidation (Aust *et al.*, 1985; Belouqui & Cederbaum, 1986; Bast & Steeghs, 1986).

In summary, these results point to the complex role for O₂ in microsomal generation of oxygen radicals. Different reaction profiles to the concentration of O₂ may be obtained, depending on the reaction being studied. Critical concentrations of O₂ are required for maximal generation of O₂^{•-} and H₂O₂, and for maintaining activity of critical enzyme components and the appropriate redox state of autoxidizable components.

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