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# CHANGES IN RAT HEPATIC MICROSOMAL MIXED FUNCTION OXIDASE ACTIVITY FOLLOWING EXPOSURE TO HALOTHANE UNDER VARIOUS OXYGEN CONCENTRATIONS

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**Abstract**—This study demonstrates that the exposure of phenobarbitone-treated rats to halothane at an oxygen concentration of either 10% or 14% results in marked decreases in cytochrome P-450 content and aminopyrine demethylase activity in animals sacrificed from 1 to 48 hr post-exposure. The alterations observed in the hepatic mixed function oxidase system were accompanied by increases in serum alanine aminotransferase (ALT), ornithine carbamyl transferase (OCT) and changes in liver pathology. However, the minor changes in cytochrome P-450 content and aminopyrine demethylase activity observed following exposure of enzyme-induced rats to halothane under normoxic conditions (i.e. 21% oxygen) were not of a sufficient magnitude to lead to hepatic cell necrosis. Halothane administration in the absence of phenobarbitone pretreatment (i.e. 21% oxygen) or during hypoxia alone (i.e either 10% or 14% oxygen) did not result in any systematic changes in the parameters assayed. The results suggest that cytochrome P-450 may catalyse its own inactivation by virtue of greater free radical production under conditions which favour the non-oxygen dependent metabolism of halothane. The impairment in microsomal function as evidenced by decreases in cytochrome P-450 and aminopyrine demethylase activity are considered to occur as a primary consequence of the reductive metabolism of halothane. Data are presented which support the concept of the initiation of hepatic damage occurring during the period of anaesthesia with halothane.

Halothane (2-bromo-2-chloro,1,1,1-trifluoroethane) is biotransformed by the hepatic mixed function oxidase enzyme system via both oxidative and reductive (or non-oxygen dependent) pathways. The oxidative metabolism of halothane to trifluoroacetic acid proceeds predominantly under normoxic conditions by the addition of activated oxygen to halothane by cytochrome P-450 [1, 2]. This pathway in the rat has been shown *not* to be associated with halothane-induced hepatotoxicity [3, 4]. However, the reductive metabolism of halothane is proportionally increased under the dual exposure conditions of reduced inspired oxygen concentration (14%) accompanied by prior induction of the drug metabolising enzyme system with phenobarbitone. Halothane administration to rats under these conditions results in extensive hepatic necrosis, the increased binding of reductive metabolites to microsomal lipid and protein *in vivo* and a decrease in cytochrome P-450 content [5, 6].

It has frequently been suggested that the metabolite which binds to microsomal macromolecules may be a free radical [7, 8]. Recently, the proposed chlorotrifluorethyl radical has been demonstrated to bind under anaerobic conditions to phospholipid in a reconstituted vesicle system containing either human or rabbit cytochrome P-450 [9]. Previous *in vitro* studies have demonstrated that reduced cytochrome P-450 forms a complex with halothane which exhibits a distinct Soret band at 470 nm. This was proposed to represent a complex between the trifluoromethyl carbene (formed as a result of two electron reduction of halothane) and the ferrous cytochrome P-450 [10, 11].

Several mechanisms, including free radicals, carbanions [12] and carbenes [10] have been proposed to explain the formation of reactive intermediates under hypoxic conditions which may then bind to cellular constituents, possibly cytochrome P-450, resulting in inactivation of this enzyme system.

There is now experimental evidence to suggest that many halogenated alkanes can undergo reductive dehalogenation by microsomal P-450 [13, 14] and a decrease in P-450 content has been reported to occur following exposure to the hepatotoxins, $CCl_4$ [15], $CBrCl_3$ [16] and $CHCl_3$ [17]. Thus a decline in cytochrome P-450 content following exposure to halothane may have implications with regard to the hepatotoxicity.

The following study was undertaken to examine the changes in the rat hepatic microsomal mixed function oxidase system following exposure to halothane *in vivo* under conditions favouring either oxidative or reductive metabolism. We have also examined whether a relationship existed between the changes observed in mixed function oxidase activity and the development of hepatic necrosis.

## MATERIALS AND METHODS

Adult male in-bred Fischer 344 rats (200–300 gm) were obtained from a breeding colony maintained at Flinders Medical Centre. Hepatic microsomal enzymes were induced prior to exposure to halothane

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by administration of 0.1% (w/v) sodium phenobarbitone in the drinking water for seven days followed by plain water for 1 day. Phenobarbitone-pretreated rats were then exposed to 1% halothane at an oxygen concentration of either 21, 14 or 10% (balance nitrogen) essentially as previously described using the glass anaesthetic mask [3]. The exposure period was 2 hr with the exception of the animals sacrificed after 1 hr or anaesthesia. Control animals received phenobarbitone and were exposed in an 8-litre glass tank to an atmosphere of oxygen at the same concentration as the respective halothane group.

Animals were sacrificed at specific times post-exposure (range 1–120 hr) and the hepatic microsomal fraction prepared by calcium aggregation [18] and analyzed for protein content [19]. Aminopyrine demethylase activity was measured using an *in vitro* incubation system consisting of 100 mM Tris–Cl buffer (pH 7.25 at 37°), 10 μmoles isocitrate, 1.2 IU isocitrate dehydrogenase, 20 μmoles magnesium chloride, 10 μmoles semicarbazide HCl, 20 mM tetrasodium pyrophosphate, 0.44 mM NADP, 4 mM aminopyrine and 2.5 mg of microsomal protein in a final volume of 2.5 ml. The reaction was stopped with perchloric acid (0.6 N, 5 ml) after 10 min incubation and the supernatant assayed for formaldehyde [20]. For the determination of aniline hydroxylation the semicarbazide HCl was omitted and aniline at a final concentration of 4 mM replaced aminopyrine as the substrate. The extent of aniline hydroxylation was assessed by the anodic oxidation of the metabolite p-aminophenol [21].

In addition, the microsomal fraction was assayed for cytochrome P-450 content [22] and NADPH cytochrome *c* reductase activity [23]. Blood was collected from the tail vein pre-exposure and at the time of sacrifice and assayed for serum alanine aminotransferase (ALT) using a Beckman TR Enzyme Activity Analyzer calibrated using quality control standards. The activity of ornithine carbamyl transferase (OCT) was also measured in pre- and post-exposure serum samples [24, 25]. These serum enzymes were used as biochemical indices of liver damage.

Liver tissue obtained *in situ* by needle biopsy (Travenol Tru-Cut biopsy needle, 20 mm specimen notch) following opening of the abdominal cavity were placed in 10% buffered formalin. Tissue samples were then embedded in Polaron Araldite sectioned at 2 microns and stained with haematoxylin and eosin (H & E) for light microscopic examination. Each section was coded and the pathologist had no prior knowledge of the treatment received by the animals.

*Statistical analysis.* Results were analysed using a three-way analysis of variance utilising the program ANOVA in the Statistical Package for the Social Sciences (SPSS) computing package. If the three-way interaction was significant, the ABC matrix was further investigated to locate the combination of variables responsible for the interaction [26]. To achieve this, the "simple interaction" effects were analysed using an analysis of unweighted cell means. If the "simple interaction" was then significant, the significance of the "simple main effects" of the three-way interaction was subsequently tested. This statistical manipulation concentrated on the "simple main effect" of one independent variable at all combinations of the other two variables.

When considering the "simple interactions" only two were relevant (i.e. halothane at oxygen by time and time at halothane by oxygen). Significance of these tests enabled subsequent analysis of the effect of halothane at a specific time point and oxygen concentration (i.e. "simple main effect"). If, however, the three-way interaction was non-significant, no further analysis was undertaken but statistical interpretation was based on any significant two-way interaction.

## RESULTS

### *Effect in non-phenobarbitone pretreated rats*

Animals exposed to halothane in the absence of enzyme induction at an oxygen concentration of 21% and sacrificed at 2 and 24 hr did not exhibit any significant alterations ($P > 0.05$) in the microsomal parameters measured when compared to control (i.e. non-halothane-exposed) animals, i.e. cytochrome P-450 content, aminopyrine demethylase (AD), aniline hydroxylase (AH) and NADPH cytochrome *c* reductase (cytc) activities were unchanged. Also, the biochemical indices of liver damage, serum ALT and OCT were not elevated by exposure to halothane. Similarly, the results obtained following the exposure of rats to halothane in an inspired oxygen concentration of 14% compared to rats exposed to 14% oxygen only indicated that halothane administration in the absence of enzyme induction and at a reduced oxygen concentration did not result in changes in either the hepatic MFO system or serum ALT and OCT.

### *Role of hypoxia*

The role of hypoxia *per se* in the production of hepatic necrosis was studied in both phenobarbitone-pretreated and untreated rats sacrificed at 2 and 24 hr. The administration of 14% oxygen for a period of 2 hr to non-induced animals did not result in alterations in P-450 content, AD, AH or cytc reductase activities when compared to the control (normoxic, i.e. 21% oxygen) rats. In addition, there were no changes in either serum ALT or OCT. Similarly, phenobarbitone-pretreated rats exposed to an atmosphere of 14% oxygen and sacrificed at 2 and 24 hr did not exhibit alterations in P-450 or AD. However, a decrease was observed in aniline hydroxylase (2 hr, $18.72 \pm 2.57$, mean $\pm$ SD) and NADPH cytochrome *c* reductase (24 hr, $116.35 \pm 7.9$) when compared to rats receiving only phenobarbitone treatment (i.e. 21% oxygen); $25.2 \pm 0.33$ nmoles p-aminophenol/mg protein/10 min and $131.63 \pm 1.16$ nmoles cytochrome *c* reduced/mg protein/minute/ml cuvette contents respectively. These changes in AH and cytc were not apparent at 24 hr and 2 hr respectively. Therefore, these latter effects were considered to be non-specific as no significant increase occurred in either serum ALT or OCT. The results suggested that hypoxia *per se* did not result in changes indicative of hepatic damage.

Table 1. Microsomal cytochrome P-450 content and aminopyrine demethylase activity in animals exposed to halothane in 21% inspired oxygen[a]

| Time[b] (hr) | Cytochrome P-450[c] Halothane | Control | Aminopyrine demethylase[c] Halothane | Control |
|---|---|---|---|---|
| 0 | — | 1.34 ±0.16 | — | 58.08 ±5.02 |
| 1 | 0.86* ±0.04 | 1.14 ±0.03 | 55.3* ±2.2 | 64.2 ±4.5 |
| 2 | 0.83* ±0.04 | 1.12 ±0.02 | 33.43* ±2.63 | 48.47 ±1.1 |
| 4 | 1.18* ±0.09 | 1.45 ±0.02 | 54.04* ±6.5 | 68.9 ±3.9 |
| 6 | 0.86** ±0.05 | 1.05 ±0.04 | 49.47* ±4.41 | 65.0 ±1.09 |
| 12 | 0.71 ±0.05 | 0.79 ±0.04 | 24.98 ±0.75 | 28.1 ±0.38 |
| 24 | 0.89 ±0.03 | 0.9 ±0.02 | 34.3 ±1.15 | 37.2 ±1.15 |
| 48 | 0.9 ±0.07 | 0.85 ±0.02 | 20.74 ±2.96 | 19.08 ±1.69 |

[a] Phenobarbitone-pretreated rats (N = 5) were exposed to 1% halothane in an inspired oxygen concentration of 21% for 2 hr (excepting animals sacrificed at 1 hr). Control rats (N = 3) received phenobarbitone only.

[b] Time represents hours sacrificed from commencement of the exposure period.

[c] Cytochrome P-450 expressed as nmoles/mg microsomal protein and aminopyrine demethylase activity as nmoles HCHO/mg microsomal protein/10 min, mean ± SD.

[d] There was a statistically significant difference between halothane exposure and control rats in cytochrome P-450 content ($F = 16.2$, $P < 0.025$**, $P < 0.001$*) and aminopyrine demethylase activity ($F = 29.6$, $P < 0.001$*).

*Time course of changes in hepatic mixed function oxidase activity*

*Cytochrome* P-450 *Content.* Administration of 1% halothane to phenobarbitone-pretreated rats at an oxygen concentration of either 21, 14 or 10% resulted in a significant reduction in cytochrome P-450 content when compared to phenobarbitone-pretreated rats exposed at the same oxygen concentration. In the group of animals exposed to halothane at 21% oxygen, microsomal P-450 content was significantly reduced ($P < 0.025$) in those animals sacrificed 1, 2, 4 and 6 hr from commencement of anaesthesia (Table 1). However, at the latter time points, i.e. 12, 24 and 48 hr, there was no significant decrease in P-450 compared to the control rats.

In contrast, phenobarbitone-treated animals exposed to halothane in an inspired oxygen concentration of 14% exhibited a significant reduction ($P < 0.001$) in P-450 content at all times of sacrifice from 1 to 24 hr post-exposure (Fig. 1A). By 48 hr, there was no significant difference in the concentration of microsomal P-450 between halothane treated and control animals exposed only to 14% oxygen. In a group of phenobarbitone pretreated rats studied 5 days post-exposure the P-450 content of halothane exposed animals, 0.94 ± 0.08 (mean ± SD) was significantly increased ($P < 0.025$) compared to phenobarbitone rats exposed to 14% oxygen only, 0.74 ± 0.05 nmoles/mg microsomal protein. The decrease in cytochrome P-450 content in rats exposed to hypoxia alone and killed at 2 and 5 days represents the decay in the inductive effects of phenobarbitone.

Similarly, animals exposed to halothane in 10% inspired oxygen showed marked decreases in P-450 content ($F = 496$, $P < 0.001$) at all times of sacrifice post-exposure (Table 2). In contrast to animals treated with halothane at 14% oxygen, cytochrome P-450 content remained significantly decreased ($P < 0.001$) in the 10% oxygen group sacrificed at 48 hr.

An overall perspective of the effect of halothane on P-450 when administered at the three oxygen concentrations is shown in Fig. 2. The use of the ratio, mean exposed/mean control value diminished the fluctuations due to both diurnal variation and the gradual decline in the inductive effect of phenobarbitone observed in presentations of the individual data (i.e. Fig. 1A, Tables 1 and 2). Cytochrome P-450 content was reduced over the initial 2-hr exposure period at 21% oxygen; however, the reduction in P-450 further increased as the concentration of oxygen was decreased from 14% to 10%. An initial recovery phase was observed immediately post-exposure at all oxygen concentrations while in the 21% group P-450 content had returned to control values by 24 hr. At the lower oxygen concentrations (10 and 14%) the gradual return towards pre-exposure values over the period 12–48 hr would appear to reflect the regenerative processes of the liver. This is further illustrated by the similarity in the regression line slopes over the 12–48 hr period post-exposure; these were 0.008 and 0.007 for the 14 and 10% groups respectively (Fig. 2). A similar slope of 0.01 was observed from 6 to 24 hr in the 21% group.

*Aminopyrine demethylase.* Aminopyrine demethylase (AD) activity was significantly decreased in phenobarbitone pretreated rats fol-

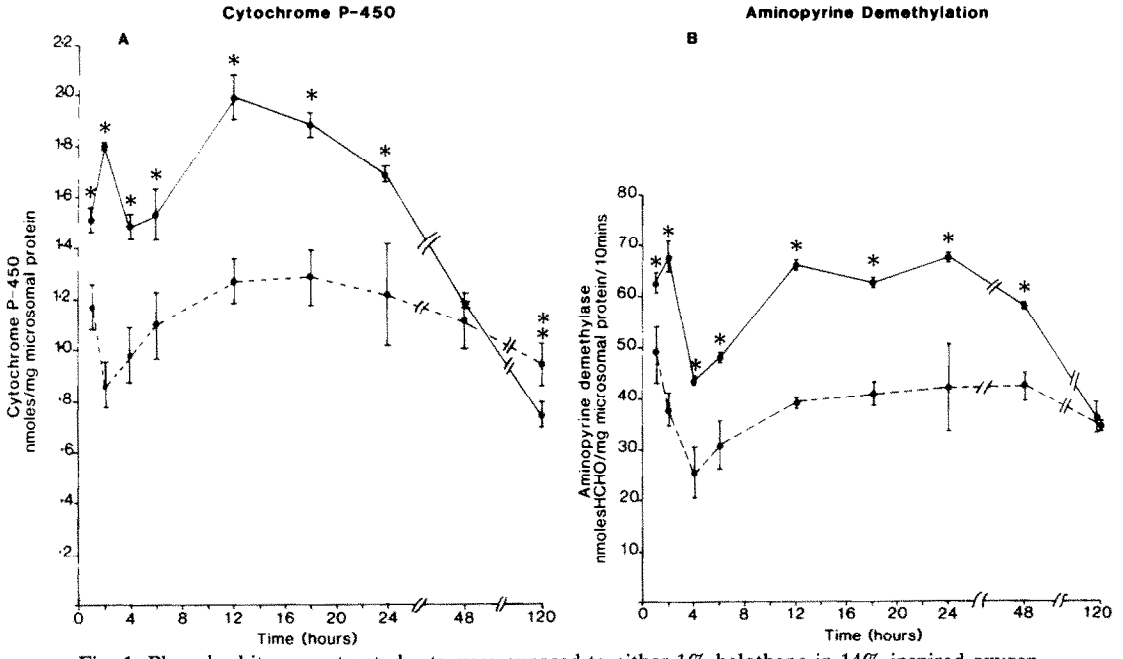


Fig. 1. Phenobarbitone pretreated rats were exposed to either 1% halothane in 14% inspired oxygen ●—● (N = 5) or to 14% oxygen only ●—● (N = 3) for a period of 2 hr. Data presented as mean ± SD. Time represents hours sacrificed from commencement of the exposure period. (A) Cytochrome P-450 content is expressed as nmoles/mg microsomal protein. Data significantly different between the groups ($F = 242$, $P < 0.001$*, $P < 0.025$**). (B) Aminopyrine demethylase activity is expressed as nmoles HCHO/mg microsomal protein/10 minutes. Data significantly different between the groups ($F = 268.3$, $P < 0.001$*).

lowing exposure to halothane at an oxygen concentration of either 21, 14 or 10%. In those animals exposed at 21% oxygen and sacrificed 1, 2, 4 and 6 hr from commencement of anaesthesia, AD activity was significantly decreased ($P < 0.001$); however, at the latter time points, 12, 24 and 48 hr, no difference in aminopyrine demethylase activity was detected in the halothane exposed compared to the normoxic animals (Table 1). In contrast, exposure to halothane in 10 or 14% oxygen resulted in a marked diminution ($P < 0.001$) in AD activity in all animals sacrificed at time points ranging from 1 to 48 hr post-exposure when compared to phenobarbitone pretreated animals exposed only to an atmosphere of either 10 or 14% oxygen (Table 2, Fig. 1B). However, in a group of animals sacrificed 120 hr from commencement of anaesthesia with 1% halothane in 14% oxygen, AD activity was not sig-

Table 2. Microsomal cytochrome P-450 content and aminopyrine demethylase activity in animals exposed to halothane in 10% inspired oxygen[a]

| Time[b] (hr) | Cytochrome P-450[c] Halothane | Control | Aminopyrine demethylase[c] Halothane | Control |
|---|---|---|---|---|
| 2 | 0.66* | 1.85 | 28.91* | 74.77 |
| | ±0.08 | ±0.07 | ±4.2 | ±0.45 |
| 6 | 0.87* | 1.51 | 23.69* | 59.68 |
| | ±0.16 | ±0.05 | ±6.43 | ±1.41 |
| 12 | 0.65* | 1.7 | 17.75* | 60.27 |
| | ±0.02 | ±0.09 | ±1.05 | ±3.95 |
| 24 | 0.72* | 1.52 | 31.31* | 80.29 |
| | ±0.05 | ±0.04 | ±1.71 | ±2.53 |
| 48 | 0.76* | 1.15 | 19.55* | 40.5 |
| | ±0.04 | ±0.04 | ±5.6 | ±2.98 |

[a] Phenobarbitone-pretreated rats were exposed to either 1% halothane in 10% inspired oxygen for 2 hr (N = 5) or to 10% oxygen only (control rats, N = 3).

[b] Time represents hours sacrificed from commencement of the exposure period.

[c] Cytochrome P-450 content expressed as nmoles/mg microsomal protein and aminopyrine demethylase activity as nmoles HCHO/mg microsomal protein/10 minutes, mean ± SD.

[d] Data were statistically significantly different between halothane exposed and control rats for cytochrome P-450 ($F = 496$, $P < 0.001$*) and aminopyrine dimethylase activity ($F = 647.2$, $P < 0.001$*).

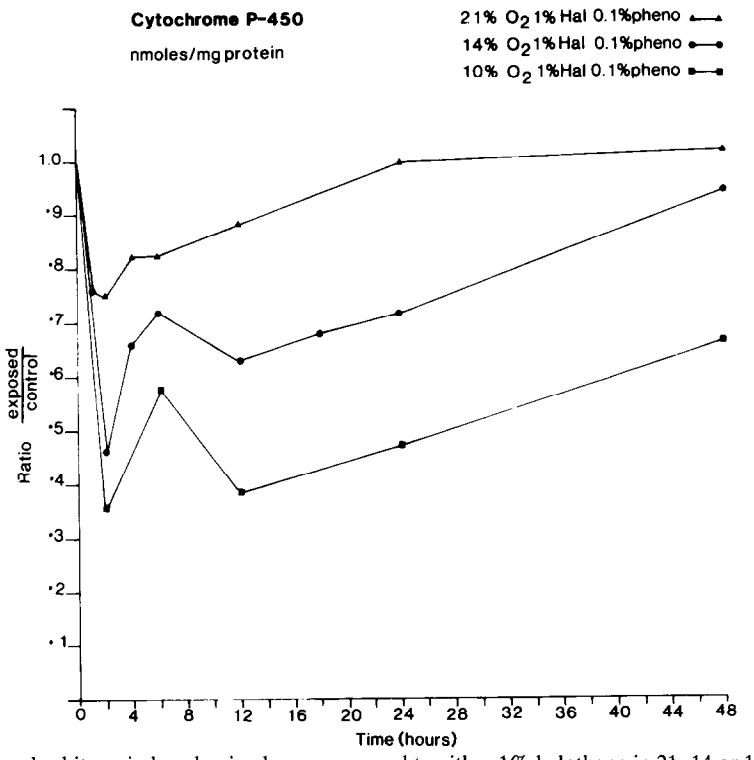


Fig. 2. Phenobarbitone induced animals were exposed to either 1% halothane in 21, 14 or 10% oxygen (exposed) or to the respective oxygen concentration only (control). Time represents hours sacrificed from commencement of the exposure period. Data are presented as the ratio of mean exposed/mean control value.

nificantly different 34.32 ± 1.64 (mean ± SD) compared to rats (36.08 ± 3.36 nmoles HCHO/mg microsomal protein/10 min) exposed to 14% oxygen in the absence of halothane (Fig. 1B) and sacrificed at the same time.

An overall perspective of the effect of halothane on AD activity when administered at 21, 14 or 10% oxygen can be obtained from Fig. 3. The ratio of mean exposed/mean control values indicated that over the 2-hr exposure period there was a decrease in AD activity, the magnitude of which increased as the oxygen concentration was lowered to 14% and 10%. With the exception of the animals exposed to halothane at 21% oxygen, AD activity remained significantly reduced over the following 46 hr post-exposure in both the 14 and 10% oxygen groups. Analogous to cytochrome P-450, the gradual return towards pre-exposure values over the 12–48-hr period would appear to reflect the regenerative processes of the liver. This is illustrated by the comparable regression line slopes of 0.005, 0.003 and 0.005 for the 21, 14 and 10% groups respectively (Fig. 3).

*Aniline hydroxylase.* In contrast to both P-450 content and AD activity, aniline hydroxylase (AH) activity in phenobarbitone-treated rats was unchanged by the administration of halothane in an inspired oxygen concentration of either 21, 14 or 10% when compared to phenobarbitone (control) animals exposed only to the appropriate oxygen atmosphere. Aniline hydroxylase activity in animals exposed to halothane at 21% oxygen, 25.01 ± 8.86 was not significantly different from the control value of 24.8 ± 6.6 nmoles PAP/mg microsomal protein/10 min (cumulative mean ± SD over all time points studied). Similarly at 14% oxygen halothane exposure, 23.6 ± 4.71 (cumulative mean ± SD over all time points studied) did not result in a reduction in AH activity when compared to the control group 25.71 ± 5.33 nmoles PAP/mg microsomal protein/10 min. An analogous situation occurred following exposure to halothane in 10% inspired oxygen, AH activity in the exposed group was 28.1 ± 3.97 (mean ± SD) compared to 34.0 ± 3.12 in the 10% oxygen treated control rats. Statistical analysis of the results indicated that there was no significant three-way interaction. However, there was a significant two-way interaction of halothane by oxygen ($F = 12.35$, $P < 0.001$), which will be discussed in a following section.

*NADPH cytochrome c reductase.* Analogous to aniline hydroxylase, NADPH cytochrome *c* reductase activity was not significantly altered by exposure to halothane at the three oxygen concentrations studied. The cumulative mean of the reductase activity at all time points studied from animals exposed to halothane in 21% oxygen was 94.33 ± 48.48 nmoles cytc reduced/mg microsomal protein/minute/ml compared to the control value of 90.3 ± 41.7 (mean ± SD). Similarly, when compared to animals exposed to 14% oxygen only (118.9 ± 13.1), no significant change occurred in the activity of NADPH cytochrome *c* reductase in those animals exposed to halothane at the same oxygen

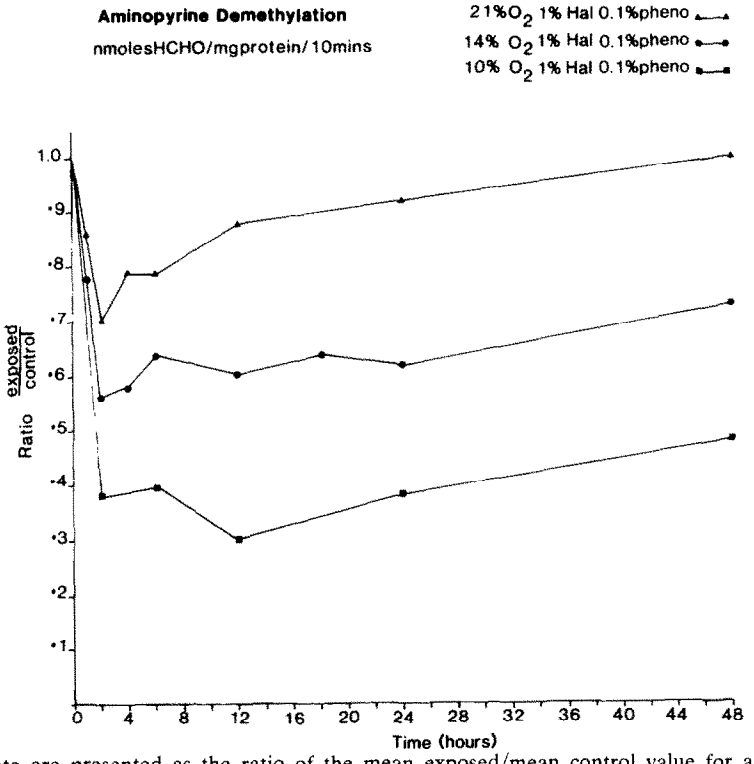


Fig. 3. Data are presented as the ratio of the mean exposed/mean control value for aminopyrine demethylation in phenobarbitone induced animals exposed to halothane at either 21, 14 or 10% oxygen (exposed) or to the respective oxygen concentration only (control). Time represents hours sacrificed from commencement of the exposure period.

concentration (121.7 ± 11.6, mean ± SD). In addition, NADPH cytochrome *c* reductase activity was not significantly altered by exposure to halothane at an oxygen concentration of 10%, 135.07 ± 20.04 when compared to the respective control group, 151.6 ± 28.44 (cumulative mean ± SD over all time points studied). Statistical analysis of the data indicated no significant three-way interaction; however, analogous to AH there was a significant two-way interaction of halothane by oxygen $F = 9.95$, $P < 0.001$. It would appear therefore that hypoxia *per se* may have increased the reductase activity.

*Maintenance of the phenobarbitone induction effect by hypoxia*

The results suggested that phenobarbitone-treated animals exposed to either 14 or 10% in the absence of halothane and sacrificed at 24 hr had increased hepatic MFO activity compared to induced rats maintained under normoxic (21%) conditions (Table 3). However, when comparing data from animals exposed to either 10 or 14% oxygen only, to a group of animals, phenobarbitone-induced and sacrificed immediately on completion of the induction period, i.e. 7 days, the results suggested a maintenance of the induced levels over the 48-hr period following the cessation of phenobarbitone administration. In contrast, animals exposed to 21% oxygen exhibited the expected decline in the inducing effects of phenobarbitone.

*Serum alanine aminotransferase and ornithine carbamyl transferase*

Rats exposed to 1% halothane in 21% oxygen and sacrificed from 1 to 48 hr post-exposure did not exhibit an increase in serum ALT above that detected in control animals (Fig. 4). However, following exposure to halothane at 14% oxygen, serum ALT was significantly increased in animals sacrificed at 18 hr, 274 ± 156 IU/l; 24 hr, 335 ± 171 IU/l ($P < 0.01$) and 48 hr, 1930 ± 652 IU/l (mean ± SD) compared to the control group, 95 ± 10, 104 ± 13 and 97 ± 12 IU/l (mean ± SD). Serum ALT was also significantly increased in animals sacrificed from 6 to 48 hr after exposure to 1% halothane in 10% inspired oxygen. At 48 hr, the mean ALT value in this group was 3181 ± 708 IU/l (mean ± SD) compared to the control group, 60 ± 9 IU/l (Fig. 4).

Statistical analyses of the data was as reported in preceding sections. The simple interaction of halothane was not evident at 21% oxygen ($F = 0.44$). However, increases in serum ALT occurred in the halothane-exposed animals at the two lower oxygen concentrations, The F values were 23.9 ($P < 0.001$) and 120 ($P < 0.001$) for the 14 and 10% groups respectively.

Similarly to serum ALT, OCT levels were unchanged from control values in rats exposed to halothane at an oxygen concentration of 21% and sacrificed from 1 to 48 hr from commencement of the exposure period. No correlation ($r = 0.4$) existed

Table 3. The effect of oxygen concentration on hepatic mixed function oxidase activity

| Oxygen concentration | P-450 | AD | AH | Cytc |
|---|---|---|---|---|
| Control | 1.62 ± 0.15 | 58.08 ± 5.02 | 30.1 ± 2.77 | 133.2 ± 9.18 |
| 21% | 0.9 ± 0.02 | 37.2 ± 1.15 | 14.4 ± 1.42 | 61.65 ± 0.63 |
| 14% | 1.69 ± 0.03* | 67.86 ± 1.25* | 20.9 ± 1.31** | 118.8 ± 7.94* |
| 10% | 1.52 ± 0.05* | 80.29 ± 2.53* | 34.4 ± 2.46* | 167.4 ± 7.63* |

Control animals were pretreated with phenobarbitone and sacrificed immediately on completion of the induction period, i.e. 7 days. The remaining animals received plain drinking water on day 8 followed by exposure on day 9 to either 21, 14 or 10% oxygen for a period of 2 hr. Animals were then sacrificed 24 hr from the commencement of the exposure period.

Cytochrome P-450 content (P-450)—nmoles/mg microsomal protein. Activity of AD, AH and cytc as previously stated.

Data presented as mean ± SD, N = 3. Significantly elevated values compared to rats exposed at 21% oxygen are denoted ($P < 0.001$*, $P < 0.01$**).

between OCT and ALT values in this group. In contrast, an increase in serum OCT concentration correlated with increasing serum ALT ($r = 0.94$) in animals exposed to halothane at 14% oxygen. A similar correlation between OCT and ATL was found in rats administered halothane in 10% inspired oxygen ($r = 0.82$). The cumulative value of OCT at all times of sacrifice in control animals exposed to 14 and 10% oxygen in the absence of halothane was 0.31 ± 0.08 and 0.35 ± 0.05 IU/l respectively (mean ± SD).

There was no statistical evidence of a three-way interaction but a significant two-way interaction of halothane by oxygen ($F = 9.078$, $P < 0.001$) occurred with regard to serum OCT. The results suggested that halothane significantly increased serum OCT when administered in an atmosphere of either 14 or 10% oxygen. A comparable result for serum ALT also indicated a significant effect of halothane ($F = 36.78$, $P < 0.001$) at the lower oxygen concentrations.

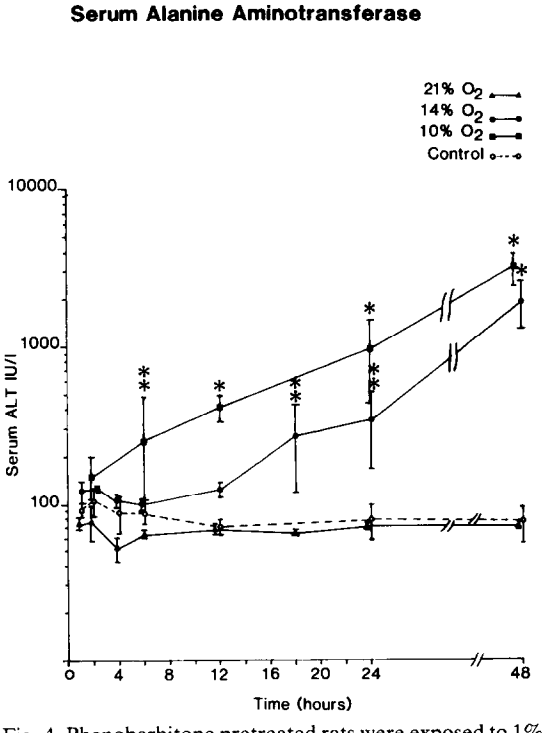


Fig. 4. Phenobarbitone pretreated rats were exposed to 1% halothane in either 21, 14 or 10% inspired oxygen (N = 5) for 2 hours. Blood was collected at the time of sacrifice and assayed for alanine aminotransferase activity. The control data represents the cumulative mean of ALT values for phenobarbitone pretreated rats exposed to either 21, 14 or 10% oxygen only. Time represents hours sacrificed from commencement of the exposure period. Data presented as mean ± SD with statistically significant difference from control values denoted as $P < 0.01$** and $P < 0.001$*. Note that ALT values are on a log scale.

*Morphological description of liver biopsy samples*

There was no evidence of liver damage in phenobarbitone pretreated animals exposed to 1% halothane in 21% oxygen and sacrificed from 1 to 120 hr post-exposure. In contrast liver biopsies examined by light microscopy from animals exposed to 1% halothane in 14% oxygen with prior phenobarbitone induction showed ballooning degeneration of liver cells in the region of the central veins. By 24 hr confluent necrosis was apparent around the central veins and the ballooning degeneration was not as prominent. A mild lymphocytic infiltrate was also present in the areas of damage. In animals sacrificed at 48 hr many necrotic cells had undergone lysis and the lymphocytic infiltrate was more pronounced.

Phenobarbitone-pretreated animals administered halothane in an inspired oxygen concentration of 10% showed signs of mild non-specific changes, i.e. hepatocyte swelling and large fat droplets in the cytoplasm as early as 6 hr post-exposure. The hepatic lesion in this group of animals progressed in a similar manner to that previously described to occur following halothane administration at 14% oxygen.

In control animals, pretreated with phenobarbitone and exposed to an atmosphere of 10% oxygen, small fat droplets were evident in the cytoplasm. It is suggested that this is a manifestation of phenobarbitone treatment as it occurred to a similar extent at all times of sacrifice.

## DISCUSSION

Recently the reductive metabolism of halothane was investigated in three rat strains, the Fischer 344,

the Sprague–Dawley and the black hooded Wistar [27]. Phenobarbitone-pretreated Fischer 344 rats compared to the other two strains exhibited greater changes in serum ALT and hepatic pathology when sacrificed 24 hr after exposure to halothane in 14% oxygen. The authors concluded that the differences between the strains may reflect a genetic basis for the formation and/or susceptibility of the liver to reactive intermediates formed during cytochrome P-450 catalysed reductive metabolism of halothane.

In this study, following exposure of phenobarbitone pretreated Fischer rats to halothane at an oxygen concentration of 14%, the activities of hepatic microsomal aniline hydroxylase and NADPH cytochrome *c* reductase were not significantly different to control animals at any of the time points studied. In accord with these results other investigators have demonstrated that halothane, when administered intraperitoneally to phenobarbitone-treated rats, had no significant effect on aniline hydroxylation in animals sacrificed at 24 hr [28].

Aniline is a type II substrate and as such forms a complex with oxidised P-450 resulting in a difference spectra [29] which is presumed to reflect a direct interaction of aniline with the ferric form of the haemoprotein, probably as a sixth ligand of the haem iron [30]. Metabolism of halothane by hepatic microsomes *in vitro* under either an air or nitrogen atmosphere has been reported not to result in a decrease in microsomal haem content [31]. It would appear that halothane may not destroy the haem moiety of cytochrome P-450 and this experimental observation may provide an explanation as to the lack of effect of halothane on aniline hydroxylase activity reported in this study. Of interest, however, is our observation from preliminary experiments that the intrinsic activity of aniline hydroxylase in the Fischer 344 rat was at a comparable level to that achieved by phenobarbitone induction in Wistar rats. In contrast to the latter strain, administration of phenobarbitone to Fischer rats did not result in a further increase in aniline hydroxylase activity suggesting that the Fischer 344 rat may have a variant of cytochrome P-450 responsible for aniline hydroxylation which is neither inducible by phenobarbitone nor affected by halothane administration. In support of this concept reports have appeared in the literature concerning the multiplicity of aniline hydroxylases in rat liver microsomes [32, 33].

Alternatively it has been proposed that aniline hydroxylase activity may be dependent on the level of cytochrome P-450 reductase activity [34]. The activity ascribed to cytochrome P-450 reductase may be assessed on the ability of the enzyme to reduce cytochrome *c* an artificial electron acceptor [35]. In this study microsomal NADPH cytochrome *c* reductase activity was unchanged by exposure of the animals to halothane under all experimental conditions.

As cytochrome $b_5$ has also been shown not to decrease following halothane administration [5], this suggests that the interaction of halothane with P-450 may be of a more selective nature involving the binding site associated more with type I substrates, e.g. aminopyrine. In contrast to the type II site, the type I site is intimately associated with phosphatidylcholine [36] and intermediates of the reductive metabolism of halothane have been demonstrated to bind to phosphatidylcholine in an anaerobic incubation system [9]. This may perturb the lipid environment surrounding P-450 and further impair its catalytic function.

In contrast to aniline hydroxylase and NADPH cytochrome *c* reductase, both cytochrome P-450 content and aminopyrine demethylase activity were decreased by exposure to halothane at all oxygen concentrations. However, in those animals exposed to halothane at 21% oxygen both parameters had returned to control values by 12 hr post-exposure. The activity of aminopyrine demethylase is dependent on the level of cytochrome P-450 [34] and therefore changes in aminopyrine demethylase activity were a reflection of alterations in cytochrome P-450 content. Further evidence in support of this concept was obtained in this study by the experimental observation of similar slopes of the data obtained from animals sacrificed from 12 to 48 hr for P-450 (Fig. 2) and aminopyrine demethylase (Fig. 3). In animals exposed to halothane at 14% oxygen, P-450 content and aminopyrine demethylase activity were decreased and lowering the oxygen concentration to 10% resulted in a further reduction in these two parameters. These results are in support of a previous study which had demonstrated a decrease in hepatic cytochrome P-450 content in rats sacrificed 6 and 24 hr after exposure to halothane in 8% oxygen [5].

In contrast, phenobarbitone-pretreated animals exposed to either 10 or 14% oxygen in the absence of halothane and sacrificed at 24 hr had enhanced activity of all microsomal parameters measured. By comparison to data obtained from animals sacrificed on completion of the phenobarbitone induction process the results suggested a maintenance effect of the induction process. This phenomenon of induction of cytochrome P-450 by hypoxia has been reported to occur in mice as early as 6 hr following exposure to low oxygen concentrations [37]. Calculations from the data provided by the authors indicated that the induction process occurred at oxygen concentrations less than 15%. The apparent sustaining of the induction effect in the control (hypoxia exposed) animals sacrificed at 24 hr was observed in other studies investigating the mechanism of halothane induced hepatotoxicity [5, 6]. Although it may not be significant with regard to the control animals, there was an apparent increase in quantifiable cytochrome P-450 content in the immediate post-exposure period (i.e. 2–6 hr) in halothane-treated animals exposed at either 14 or 10% oxygen (Fig. 1A, Table 2). This may have some implication with regard to the metabolism of halothane released from adipose tissue stores in the post-exposure period [38].

Following exposure of phenobarbitone-pretreated rats to 1% halothane at 14% oxygen, gross fatty changes have been reported to occur in the livers of animals sacrificed at 24 hr [27]. Serum ornithine carbamyl transferase may be elevated in situations of fatty infiltration of the liver [39] and for this reason it was possible that OCT activity may have been a more sensitive index of liver damage following

exposure to halothane. In the present study, decreases in hepatic mixed function oxidase activities were accompanied by increases in both serum alanine aminotransferase and ornithine carbamyl transferase. Elevations in the serum enzyme concentrations were more extensive in enzyme-induced animals exposed to halothane in 10% inspired oxygen while hypoxia alone or hypoxia plus halothane anaesthesia did not result in increases in either ALT or OCT (Fig. 4). Although a correlation was observed between increasing serum ALT and OCT the latter did not appear to be a more sensitive index of parenchymal damage.

Serum ALT was significantly increased in those animals exposed to halothane at 14% oxygen and sacrificed at 24 hr when compared to phenobarbitone-pretreated hypoxia-exposed rats. In conjunction with the elevated serum enzymes, hepatic necrosis was observed in the area of the central veins in biopsy samples obtained from these animals. Rats exposed to halothane at 10% oxygen showed evidence of mild liver changes as early as 6 hr post-exposure and the pathology findings were supported by the observation of an increase in serum ALT in the same animals.

The changes observed in cytochrome P-450 content and aminopyrine demethylase activity following exposure of enzyme induced rats to halothane under normoxic conditions were not of a sufficient magnitude to lead to hepatic cell necrosis. The anaesthetic state *per se* would appear not to be responsible for the minor alterations in hepatic mixed function oxidase activity as decreases in P-450 and aminopyrine demethylase were not observed in the absence of enzyme induction. It is possible that an oxidative metabolite, e.g. trifluoroacetyl chloride may bind to P-450 to form a reversible complex. Recent *in vivo* and *in vitro* evidence has suggested that the trifluoroacetyl chloride intermediate is not a causative factor in halothane-induced hepatic necrosis [2]. The results of this study are in accord with previous investigators who have demonstrated that the oxidative metabolism of halothane appears not to be implicated in the production of hepatic necrosis [4–6].

However, it is apparent that intermediates of halothane metabolism produced via the reductive pathway by hepatic microsomal cytochrome P-450 may interact directly with cellular constituents of the endoplasmic reticulum [9, 11, 40]. Increased binding of $^{14}$C-halothane equivalents has been reported to occur *in vivo* and *in vitro* under conditions of reduced oxygen concentration [6, 41, 42]. The interaction of reactive intermediates, e.g. the proposed carbanion [12] or trifluoromethyl carbene [10] with microsomal components, may account for the decrease in cytochrome P-450 content and aminopyrine demethylase activity observed in rats following exposure to halothane at either 14 or 10% oxygen.

Recently a pronounced decrease in cytochrome P-450 content was demonstrated following anaerobic incubation of halothane with hepatic microsomes isolated from phenobarbitone-pretreated rats [43]. The extent of P-450 reduction was dependent on the oxygen concentration and the inactivation was virtually eliminated at an oxygen partial pressure of approximately 40 mm Hg. The results suggested that the phenobarbitone-inducible form of cytochrome P-450 may catalyse its own inactivation by virtue of the production of radical intermediates during the reductive metabolism of halothane.

It is proposed that as the oxygen concentration is decreased from 21 to 14% the proportion of halothane metabolised via the reductive pathway is increased. However, a further reduction in oxygen availability (i.e. 10%) results in a greater proportion of reductive metabolism (at the expense of oxidative metabolism) and hence a quantitative increase in reactive intermediate production. If, as has been suggested, cytochrome P-450 catalyses its own inactivation by virtue of greater free radical production, then a substantial decrease in P-450 content would be expected at the lowest oxygen concentration. In support of this concept the results of this study demonstrated that cytochrome P-450 content was decreased by approximately 52% at the end of a 2-hr exposure with halothane at 14% oxygen and 64% after the same duration of exposure at 10% oxygen.

An alternative hypothesis has suggested that cytochrome P-450 may *not* be destroyed *in vitro* by reactive intermediates of halothane metabolism [31]. These reactive intermediates may merely prevent the formation of the carbon monoxide complex which is essential for the quantitation of cytochrome P-450. However, the persistent decrease in measurable P-450 content as observed in this study over a period of 48 hr would suggest that *in vivo* cytochrome P-450 may be destroyed. The gradual return towards pre-exposure values over a period of 5 days is suggestive more of a regenerative process. In support of this concept, in animals exposed to halothane at 14% oxygen, P-450 content and aminopyrine demethylase activity had returned to control values by 120 hr at which time the lesion has almost resolved [44].

The results of this study suggest the microsomal damage as evidenced by decreases in cytochrome P-450 and aminopyrine demethylase is a primary effect of the reductive metabolism of halothane. These perturbations in microsomal function occurred before evidence of generalised cell damage. Further, the effect of hypoxia *per se* was investigated and in accord with previous reports [5, 6, 45] it would appear that in the absence of halothane it is not a direct causative factor in the development of hepatic damage. In a recent report, Van Dyke *et al.* [46] demonstrated that ornithine decarboxylase an enzyme involved in cell proliferation was increased 2–4 hr post-exposure to halothane in 10% oxygen. The authors suggested that the damage may occur in the early phases of the post-exposure period or alternatively, during the actual time of administration of halothane [47]. The data presented in this study lends support to the concept that the initiation of damage occurs intra-exposure. This hypothesis is supported by the evidence of (a) an initial decrease in cytochrome P-450 content following exposure to halothane at 14% oxygen for 1 hr with a further reduction in P-450 content occurred in phenobarbitone-pretreated rats exposed to halothane at arbitone-pretreated rats exposed to halothane at 10% oxygen and sacrificed at 2 hr. In this latter group of animals hepatic damage as assessed by serum

enzymes was evident by 6 hr post-exposure.

Other compounds metabolised by cytochrome P-450 have been shown to mediate its destruction, e.g. fluroxene [48], vinyl chloride [49] and carbon tetrachloride [50]. It is conceivable that free radicals produced as a result of the reductive metabolism of halothane may destroy cytochrome P-450 in an analogous way to the trichloromethyl radical of carbon tetrachloride.

Additional *in vitro* experimental evidence has been provided [51] which demonstrated a decrease in P-450 content following aerobic incubation of halothane with hepatic microsomes isolated from phenobarbitone pretreated rats. However, the reduction in P-450 was completely reversed by either dialysis or the addition of potassium ferricyanide. In contrast, when incubations were performed under anaerobic conditions ($N_2$) an irreversible decrease in measurable cytochrome P-450 content occurred.

Data presented in this study have indicated that the administration of halothane *in vivo* in an inspired oxygen concentration of 21% to phenobarbitone-pretreated rats resulted in transient alterations in hepatic mixed function oxidase activity which were of insufficient magnitude to lead to hepatic necrosis. In contrast, exposure to halothane in an inspired oxygen concentration of either 10 or 14% resulted in an apparent destruction of cytochrome P-450 and the subsequent production of hepatic necrosis. Thus it would appear that an analogous situation with regard to the metabolism of halothane and the subsequent interaction with P-450 occurs both *in vivo* and *in vitro*.

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