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REDUCTIVE ACTIVATION OF HALOTHANE BY HUMAN HAEMOGLOBIN RESULTS IN THE MODIFICATION OF THE PROSTHETIC HAEM

ROBERTO TOLANDO, STEFANO CAZZARO, ROBERTA FERRARA, MICHELA REZZADORE and MAURIZIO MANNO*

Institute of Occupational Medicine, University of Padua Medical School, via Facciolati 71, 35127 Padova, Italy

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Abstract—The metabolic activation of halothane by human haemoglobin (Hb) under reducing conditions *in vitro* is reported. Absolute spectra of sodium dithionite-reduced Hb, recorded during its anaerobic incubation in the presence of the substrate, showed decreasing concentrations of reduced Hb (Hb^{2+}) with time. The loss of Hb^{2+} was accompanied, although only to some extent, by a concurrent oxidation to methaemoglobin (Hb^{3+}), suggesting that electron transfer from Hb to the substrate had occurred. Reductive halothane metabolism was observed under these conditions as indicated by a dose-dependent inorganic fluoride (F^-) production, which was, however, lower than that observed with heated Hb or a water soluble haem preparation (methaemalbumin). A rapid, partial loss of Hb was found upon addition of the substrate to the incubation mixture, as indicated by a decrease of the typical peak at 418 nm in the absolute spectra recorded in the presence of carbon monoxide (CO). This effect was associated with a loss of the Hb prosthetic group, haem, as shown by a decrease of the pyridine–haemochromogen reaction. Both effects were time and dose dependent. The inhibition of the Hb inactivation reaction by adding exogenous CO or the spin trapping agent *N*-*t*-butyl-α-phenylnitrone (PBN) to the incubation mixture beforehand indicated that (a) a reduced and free haem iron is required by Hb to activate halothane, and (b) the formation of free radical reactive metabolites of halothane is likely to be responsible for Hb inactivation. The mechanism of the reaction may involve the attack of these metabolites on the haem group of Hb, as indicated by the detection, with a reverse-phase ion-pairing HPLC system, of two Hb-derived products showing a typical haem-like absorption spectrum. The present results resemble those obtained recently with carbon tetrachloride (Ferrara *et al.*, *Alternatives to Laboratory Animals* **21**: 57–64, 1993) and suggest a common mechanism of activation of the two polyhalogenated alkanes by Hb.

Key words: Haem; haemoglobin; halothane; inorganic fluoride; reductive metabolism; haemoprotein inactivation

Haemoglobin has the ability to catalyse the biotransformation of various classes of xenobiotics, both oxidatively and reductively [1, 2]. In some cases, the metabolic process may be associated with a loss of the haemoprotein itself, due to the formation of reactive metabolites at the very site of catalysis, resulting in the suicidal inactivation of the prosthetic haem group. Recently, in our laboratory, it has been shown that human Hb† is able to metabolize reductively the hepatotoxic industrial solvent carbon tetrachloride (CCl_4) to carbon monoxide (CO) [3]. During this reaction, Hb was found to suffer an irreversible modification of the prosthetic haem group. It was also found to activate trichlorobromomethane ($BrCCl_3$), another halogenated alkane known to form the same reactive species as CCl_4, i.e. the trichloromethyl radical ($\cdot CCl_3$). The reaction resulted in the formation of two protein-bound haem adducts, CCl_2–haem and CCl_3–haem [4]. A substrate-dependent loss of haem has also been reported when a number of other haemoproteins, both natural (P450 and myoglobin) and synthetic (MHA), were incubated, under anaerobic conditions and in the presence of a reducing agent, with $BrCCl_3$ or CCl_4 [5–8].

The metabolic activation by liver microsomes of another polyhalogenated alkane, the hepatotoxic anaesthetic halothane ($CF_3CHBrCl$), under anaerobic reducing conditions, also resulted in the irreversible loss of the activating enzyme P450 [9, 10]. The enzyme loss is probably due to the attack of the haem group by reactive metabolites, probably free radicals, resulting in P450 "suicidal" inactivation. The halothane-dependent inactivation of P450 haem observed in the rat [11] and the human liver preparation [12] is similar, as regards the mechanism of the reaction, to that observed with CCl_4 [7, 12].

The aim of the present study was to investigate whether human haemoglobin can also activate halothane reductively and whether the reaction, like that catalysed by P450, may result in the inactivation of the haemoprotein and loss of its prosthetic haem group. Furthermore, the reactive metabolites responsible for Hb inactivation and the modified

* Corresponding author. Tel. 39 49 821 6647; FAX 39 49 821 6603.

† *Abbreviations*: Hb, haemoglobin; MHA, methaemalbumin; NADH, β-nicotinamide adenine dinucleotide; P450, cytochrome P450; PBN, *N*-*t*-butyl-α-phenylnitrone; PMS, phenazine methosulfate; TBA, tetrabutylammonium hydroxide.

haem products formed by the reaction were investigated. A preliminary report of some of the present data has been made [13].

MATERIALS AND METHODS

Chemicals and biochemicals. Halothane (1,1,1-trifluoro-2,2-chlorobromoethane) was obtained from Aldrich Chemical Co. Ltd (Gillingham, Dorset, U.K.). NADH, NADH-cytochrome c reductase (EC 1.6.99.3), catalase (EC 1.11.1.6), PBN and glucose oxidase (EC 1.1.3.4) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Methanol was of HPLC grade, and was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). TBA and PMS were from Aldrich Chemie (Steinheim, Germany) and O_2-free nitrogen was from SIO (Milano, Italy). Other reagents were of analytical grade. Hb was prepared from human blood by a modification of the method of Rossi-Fanelli and Antonini [14]. Methaemalbumin (MHA) was prepared from haemin and human albumin (both purchased from Sigma Chemical Co.) as described by Tenhunen *et al.* [15].

Anaerobic incubations. All incubations were performed in rubber-stoppered 5 mL glass tubes when the fluoride analysis, the pyridine–haemochromogen assay and the porphyrin fluorescence technique were carried out, or in 3 mL, 1 cm light path spectrophotometer cells when Hb was measured. In order to ensure anaerobic conditions throughout incubation, care was taken to remove oxygen from the buffer and the incubation vessel by flushing O_2-free N_2 through the rubber stopper, as described elsewhere [7], and including in the incubation mixture the following oxygen scavenging system (final concentration in parentheses): catalase (600 U/mL), glucose oxidase (12.5 U/mL) and D-glucose (60 mM). Under these conditions the O_2 concentration in the incubation mixture was kept below the lowest limit of detection of the O_2 electrode. The incubation mixture contained the following concentrations of Hb: 1.8–3.9, 37.3 and 49.9 μM when spectrophotometric assays, HPLC analysis and inorganic fluoride (F^-) determination were carried out, respectively. Hb was added in a 0.1 M Na_2HPO_4 buffer, pH 7.4, in the presence of the O_2-scavenging system described above. The incubation mixture was preincubated for 10 min at 37° before the reaction was started by the addition of halothane in methanol (1–20 mM final concentration) and one of the following reducing systems: (a) a sodium dithionite solution in 0.1 M Na_2HPO_4 buffer, pH 9 (1 mM final concentration); (b) a water solution of NADH and NADH-cytochrome c reductase (1 mM and 3 U/mL final concentration, respectively); or (c) a water solution of PMS and ascorbic acid (2.5 μM and 0.25 mM final concentration, respectively). Incubation times for each experiment are reported in the legends to tables and figures.

Assays. Hb was measured by the typical 418 nm peak ($\varepsilon_{mM} = 154\ mM^{-1}\cdot cm^{-1}$) in the absolute spectrum of its complex with exogenous CO bubbled to saturating concentrations through the incubation mixture (one bubble per sec for 30 sec) in the presence of excess sodium dithionite. When necessary, the concurrent measurement of Hb^{2+} and Hb^{3+} was carried out in the same anaerobic incubation by recording the absolute reduced spectrum between 380 and 460 nm at various times after the addition of halothane and sodium dithionite. No qualitative or quantitative change in the absolute spectrum of Hb was observed upon addition of up to 20 mM halothane in the absence of the reducing agent. Based on the assumption that only these two forms of Hb were present in significant amounts in the incubation mixture, the concentration of each form was obtained by the following algebraic calculations:

$$M = \frac{A_1 \cdot r_1 - A_2 \cdot r_2}{m_2 \cdot r_1 - r_2 \cdot m_1}$$

$$R = \frac{A_2 - M \cdot m_1}{r_1}$$

where $M = [Hb^{3+}]$; $R = [Hb^{2+}]$; A_1 = absorbance at 405 nm; A_2 = absorbance at 430 nm; $m_{1,2} = \varepsilon_{mM\ 430,405\ nm}$ for Hb^{3+}; $r_{1,2} = \varepsilon_{mM\ 430,405\ nm}$ for Hb^{2+}; and using (a) the values of absorbance at the wavelength of maximum absorbance for each component (430 and 405 nm for Hb^{2+} and Hb^{3+}, respectively) as observed in the typical absolute spectrum of each species separately, and (b) the extinction coefficients of each component above (112 and 54 for Hb^{2+}, and 38 and 114 for Hb^{3+} at 430 and 405 nm, respectively) calculated as reported elsewhere [3].

Haem was measured by two different methods: the pyridine–haemochromogen method [16], using the absolute reduced spectrum and the $\varepsilon_{mM\ (557-541)} = 20.7\ mM^{-1}\cdot cm^{-1}$ [17], and the porphyrin fluorescence technique, after conversion of haem to protoporphyrin IX in saturated oxalic acid at 100° [18].

Inorganic fluoride production was determined in the incubation mixture by potentiometric measurement, after dilution in Orion low-level TISAB buffer, using a fluoride specific electrode (Orion, mod. 94-09) and a reference electrode (Orion, mod. 90-01). Appropriate calibration curves were carried out immediately before each measurement, using known solutions of NaF prepared from an Orion 0.1 M standard solution and diluted in an equal volume of low level TISAB buffer, the lowest level of detection being 1 μM.

When the protective effect of CO and PBN against the substrate-dependent loss of Hb and Hb-derived haem was investigated, CO was bubbled through, or a water solution of PBN was injected into the incubation mixture before the addition of the substrate and/or the sodium dithionite solution.

HPLC method. A slight modification of a previously described reverse-phase ion pairing method [8] was used to investigate the haem products obtained during incubation of Hb with halothane. At the end of the incubation time, 400 μL of the incubation mixture was added to 100 μL of a 4:1 (v:v) methanol–water mixture containing 25 mM TBA. After centrifugation for 2.5 min at 13,400 *g* to remove any precipitate, 20 μL of the supernatant was injected into a reverse-phase column (Spherisorb S5 OD52, 5 μm particles; 25 cm × 4.6 mm internal

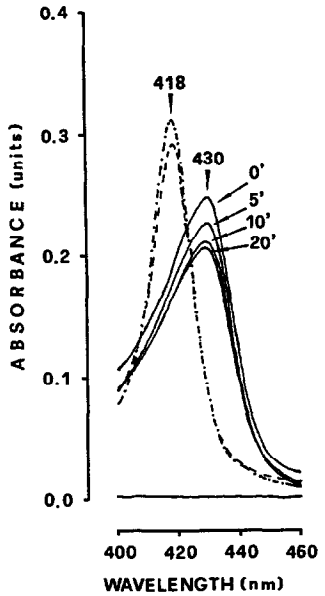

Fig. 1. Absolute reduced spectra of human haemoglobin during anaerobic incubation with halothane. The incubation was made anaerobic as described in Materials and Methods and contained 2.10 ± 0.01 (mean ± SD) nmol Hb/mL. After preincubation, 1 mM sodium dithionite was injected immediately before the addition, at time 0, of 10 mM halothane (final concentration). Absolute spectra were recorded at the indicated times (——). At time 22 min, an additional spectrum was recorded immediately after saturation of the incubation mixture with CO (---). A final spectrum was recorded at time 24 min, 1 min after further addition of sodium dithionite (·····). Further details are given in the Results section.

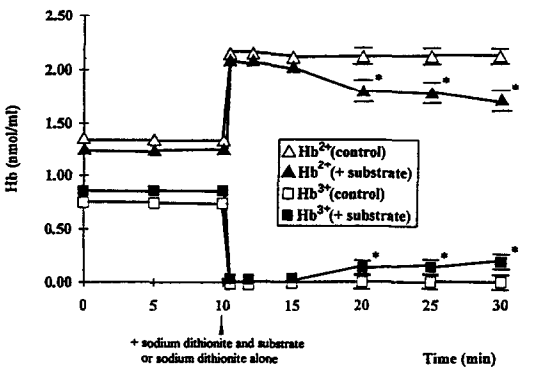

Fig. 2. Time-course of Hb^{2+} and Hb^{3+} concentration during reductive metabolism of halothane by human haemoglobin. Incubations were as for Fig. 1. The concentrations of Hb^{2+} and Hb^{3+} were calculated from the absolute spectra recorded at the indicated times and using the algebraic system and the extinction coefficients reported in Materials and Methods. *$P < 0.01$ vs control (Student's t-test). Values are the mean ± SD of three determinations.

diameter; Phase Separations Ltd, Deeside, U.K.) and eluted with a gradient elution system, using the following solvents: 35% methanol in water containing 2.5 mM TBA (solvent A) and 95% methanol in water containing 1 mM TBA (solvent B). Elution was with 100% solvent A for 1 min, with a linear gradient to 25% solvent A in the solvent A + B mixture in 7.5 min, and this latter concentration was then maintained for 7.5 min. Finally, a linear gradient to 100% solvent A was performed during the last 4 min, and these conditions were maintained for at least 4 min before the next injection. When necessary, absorption spectra of haem and haem products were recorded during the elution of each peak.

RESULTS

Upon addition of halothane to a typical anaerobic sodium dithionite-reduced incubation mixture containing Hb, the following changes in the absolute spectrum were observed with time (Fig. 1): (a) the typical peak at 430 nm, given by Hb^{2+} in the absence of the substrate, showed a significant dose-dependent (data not shown) and time-dependent decrease; (b) upon subsequent saturation of the incubation mixture with exogenous CO (approximately 1 mM final concentration), a typical 418 nm peak was formed immediately, which increased further after addition of a supplementary amount of the reducing agent (sodium dithionite solution), suggesting that part of the Hb had been oxidized to methaemoglobin. The concentration of each of these two forms of Hb in the incubation mixture was calculated from the absolute spectra recorded during the reaction, using the extinction coefficients and the algebraic system described in Materials and Methods. The time-course of Hb^{2+} and Hb^{3+} levels measured under these conditions, in the presence of 10 mM halothane, is reported in Fig. 2. On addition of the substrate the level of Hb^{3+} showed an increase, while that of Hb^{2+} showed a decrease, in a time-dependent manner. No statistically significant formation of Hb^{3+} or decrease of Hb^{2+} was observed in the absence of the substrate (Fig. 2) or the reducing agent (results not shown).

In order to test whether Hb was able to metabolize halothane reductively under the above conditions, the formation of inorganic fluoride, a well-known reductive metabolite of halothane, was investigated (Table 1). A dose-dependent production of F^- was observed during incubation of halothane with Hb in the presence of sodium dithionite, but not in the presence of the substrate or the reducing agent alone or in the absence of Hb. Carbon monoxide significantly decreased, whereas heated Hb significantly increased F^- production under the same conditions.

When the total recovery of Hb at the end of incubation was measured by recording its absolute spectrum after saturation with exogenous CO and reduction with excess sodium dithionite, as described above, a small but statistically significant substrate-dependent inactivation of Hb was found (Table 2). No statistically significant loss of Hb was observed in the absence of the substrate or the reducing agent, indicating that reductive activation of halothane to reactive metabolites is needed for the disappearance

Table 1. Inorganic fluoride production during reductive metabolism of halothane by human haemoglobin

Incubation	F^- nmol/mL
Hb	2.67 ± 0.66
Hb + dithionite	2.43 ± 0.48 NS
Hb + halothane 10 mM	3.02 ± 0.26 NS
Dithionite + halothane 10 mM	2.88 ± 0.48 NS †
Hb + dithionite + halothane 1 mM	3.06 ± 0.43 §
Hb + dithionite + halothane 5 mM	4.97 ± 0.30 *
Hb + dithionite + halothane 10 mM	7.20 ± 0.38 *
Hb + CO + dithionite + halothane 10 mM	3.31 ± 0.19 †
Heated Hb + dithionite + halothane 10 mM	39.62 ± 2.24 †

NS, not significant vs Hb alone; § not significant, and * $P < 0.001$ vs Hb + dithionite; † $P < 0.001$ vs Hb + dithionite + halothane 10 mM; Student's *t*-test.

Incubations were made anaerobic as described in Materials and Methods and contained, where indicated, 49.9 ± 1.0 (mean ± SD) nmol Hb/mL. After preincubation, the reaction was started by addition of the indicated final concentrations of halothane and/or 1 mM sodium dithionite. After 30 min, 2 mL of the incubation mixture was pipetted into a plastic beaker containing 10 mL of TISAB buffer, 25 μL of a 10^{-3} M F^- standard solution and bidistilled water, to obtain a final volume of 15 mL in which F^- determination was performed. When indicated, CO was bubbled through the incubation mixture immediately before the addition of the substrate and the reducing agent, or Hb heated at 100° for 5 min was used instead of Hb. Values are mean ± SD of four determinations.

Table 2. Halothane-dependent reductive inactivation of human haemoglobin

Incubation	Hb nmol/mL	Hb (%)	% loss
Hb	1.99 ± 0.02	(100)	0.0
+ dithionite	1.95 ± 0.02 NS	(98.0)	2.0
+ halothane	1.87 ± 0.04 NS	(94.0)	6.0
+ dithionite and halothane	1.73 ± 0.02*	(86.9)	13.1

NS, not significant vs Hb; * $P < 0.001$ vs Hb + dithionite; Student's *t*-test.

Incubations were made anaerobic as described in Materials and Methods and contained 2.02 ± 0.02 (mean ± SD) nmol Hb/mL. After preincubation, the reaction was started by addition of 10 mM halothane and/or 1 mM sodium dithionite. After a 15 min incubation, Hb was determined by the absorbance at 418 nm of the Hb–CO complex in the absolute reduced absorption spectrum recorded after addition of saturating concentrations of exogenous CO and excess sodium dithionite. Values are the mean ± SD of five determinations.

Table 3. Dose-dependent loss of human haemoglobin and haemoglobin-derived haem by halothane

Incubation	Hb nmol/mL	Hb % loss	Haem nmol/mL	Haem % loss
Control	1.64 ± 0.05	0.0	1.69 ± 0.05	0.0
+ halothane 1 mM	1.62 ± 0.03 NS	1.2	1.62 ± 0.02 NS	4.1
+ halothane 5 mM	1.48 ± 0.03*	9.8	1.48 ± 0.03*	12.4
+ halothane 10 mM	1.38 ± 0.05*	15.9	1.33 ± 0.05*	21.3

NS, not significant; * $P < 0.001$ vs control; Student's *t*-test.

Incubations were made anaerobic as described in Materials and Methods and contained 1.76 ± 0.04 (for Hb determination) and 1.80 ± 0.10 (for haem determination) nmol Hb/mL. After preincubation, the reaction was started by the addition of 1 mM sodium dithionite alone (control) or sodium dithionite and the indicated final concentrations of halothane. After incubation for 15 min, Hb was determined as described in Table 2 and Hb-derived haem was measured by the haemochromogen–pyridine method. Values are the mean ± SD of five determinations.

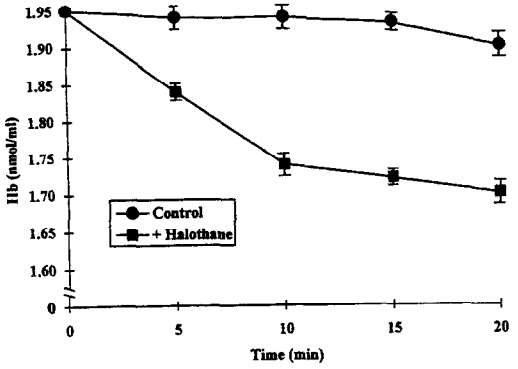

Fig. 3. Time-course of the halothane-dependent inactivation of human haemoglobin. Incubations were made anaerobic as described in Materials and Methods and contained 2.00 ± 0.04 (mean ± SD) nmol Hb/mL. After preincubation, 10 mM halothane and 1 mM sodium dithionite (final concentrations) were added to start the reaction. At the indicated times, Hb was determined as described in Table 2. Values are the mean ± SD of four determinations.

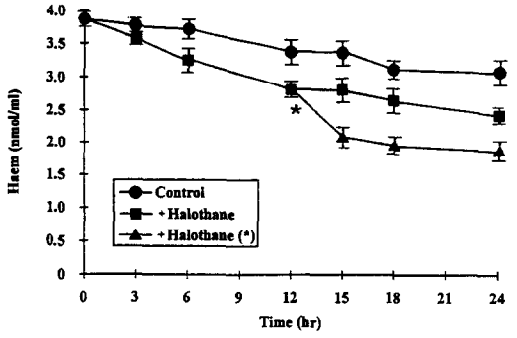

Fig. 4. Time-course of the halothane-dependent loss of human haemoglobin-derived haem in the absence of exogenous reducing agent. Incubations were made anaerobic as described in Materials and Methods and contained 3.90 ± 0.05 (mean ± SD) nmol Hb/mL. After preincubation, 10 mM halothane (final concentration) was added at time 0 to start the reaction and, to a parallel set of incubations, also at time 12 hr (*). At the indicated times haem was measured by the haemochromogen–pyridine method. Values are the mean ± SD of four determinations.

of Hb. The loss of the haemoprotein was dose dependent (Table 3) and time dependent (Fig. 3). Furthermore, when the haem content was measured by the pyridine–haemochromogen method in identical incubation mixtures, a dose-dependent (Table 3) loss of Hb-derived haem was also observed upon addition of the substrate. This loss was also time dependent (results not shown). These results suggest overall that inactivation of the haemoprotein is due to loss of the prosthetic haem group.

To confirm the critical role of haemoprotein reduction in the process, as suggested above, two additional reducing systems, ascorbic acid + PMS and NADH + NADH-cytochrome c reductase, were tested and compared with sodium dithionite regarding their ability to support the reaction (Table 4). Both these additional reducing systems caused a statistically significant loss of haem, although lower than that supported by sodium dithionite. Furthermore, since in preliminary experiments carried out in the absence of sodium dithionite, it was found that: (a) in the anaerobic environment produced by the O_2 scavenging system Hb^{3+} was slowly reduced to Hb^{2+} (results not shown), and (b) some, although statistically not significant, substrate-dependent loss of Hb and Hb-derived haem was observed (Tables 2 and 4, respectively), Hb was also measured after incubation for 24 hr with halothane but without any exogenous chemical or biochemical reducing agent. A progressive loss of Hb was found

Table 4. Effect of various reducing agents on the halothane-dependent loss of human haemoglobin-derived haem

Incubation	Haem nmol/mL	%
Hb	1.93 ± 0.03	100
+ halothane	1.89 ± 0.00 NS	97.9
+ dithionite	1.89 ± 0.05 NS	97.9
+ dithionite and halothane	1.56 ± 0.10 †	80.8
+ ascorbic acid/PMS	1.77 ± 0.03 *	91.7
+ ascorbic acid/PMS and halothane	1.56 ± 0.03 †	80.8
+ NADH/NADH cyt. c reductase	1.79 ± 0.04 *	92.7
+ NADH/NADH cyt. c reductase and halothane	1.57 ± 0.02 †	81.3

NS, not significant; * $P < 0.01$, vs Hb alone; † $P < 0.001$ vs reducing agent alone; Student's *t*-test.

Incubations were made anaerobic as described in Materials and Methods and contained 2.00 ± 0.06 (mean ± SD) nmol Hb/mL. After preincubation, the reaction was started by addition of 10 mM halothane and 1 mM sodium dithionite, or 0.25 mM ascorbic acid + 2.5 μM phenazine methosulfate, or 1 mM NADH + 3 U/mL NADH-cytochrome c reductase. After a 15 min incubation, Hb-derived haem was measured by the haemochromogen–pyridine method. Values are the mean ± SD of five determinations.

under these conditions, suggesting that some activation of halothane does occur even in the absence of exogenous reductants, providing that weak reducing conditions are maintained throughout incubation and the substrate is not limiting. Upon injection at time 12 hr of an additional equal amount of halothane, a further haem loss was achieved, indicating that after a 12 hr incubation no more substrate was available (Fig. 4).

In order to demonstrate whether interaction of the substrate with the iron of the haem group was required for Hb inactivation, we investigated the effect of CO on the reaction. A 93 and 90% protection against the halothane-dependent inactivation of Hb was provided by saturating the sodium dithionite-reduced incubation mixture with CO before the addition of 5 and 10 mM halothane, respectively (results not shown). This strongly suggests that binding of halothane to a free and reduced haem iron is required for metabolic activation of the substrate to occur.

To gain more information on the reactive species involved in the reaction, we investigated the effect of the spin trapping agent PBN. The presence of PBN in the incubation mixture under the usual conditions was associated with a lower substrate-dependent haem loss than that measured in control incubations (Table 5). This suggests that a free radical intermediate of halothane may be responsible for the Hb inactivation reaction.

Finally, in an attempt to clarify the mechanism of the haem loss, the fate of the haem tetrapyrrolic structure and the type and extent of haem modification after incubation with halothane were investigated using two additional methods: a fluorescence technique for detection of protoporphyrin IX, the iron-free precursor of haem, and the reverse-phase HPLC method described in Materials and Methods. No statistically significant substrate-dependent loss of porphyrin was observed with the fluorescence technique (results not shown), suggesting that Hb haem, unlike that of P450 [11], was not subject to destruction of its tetrapyrrolic structure. Preliminary HPLC results carried out under typical experimental conditions (anaerobic incubation of Hb with halothane in the presence of sodium dithionite) confirmed the haem loss observed with the pyridine–haemochromogen technique (Fig. 5). The area of the haem peak showed a decrease of approx. 23% as compared to control incubations containing the reducing agent but no substrate. Moreover, at least two substrate-dependent products were observed in the chromatogram which (a) absorbed at approx. 400 nm, (b) were eluted just before the haem peak, and (c) showed a haem-like spectrum with a maximum absorbance at approx. 406 nm, similar to that of authentic haem (results not shown).

DISCUSSION

Haemoglobin is known to share a number of different features with cytochrome P450. Among these are the haemoprotein structure, NADH and NADPH as cofactors for physiological reduction, O_2 as the main ligand or substrate and, finally, several catalytic activities, including N- and O-dealkylation, N-hydroxylation and aliphatic and aromatic hydroxylation [1]. Besides these monooxygenase activities, P450 can also catalyse reductive reactions, such as those responsible for the metabolism of two polyhalogenated hydrocarbons, CCl_4 and halothane [19–21]. Recent work in our laboratory has shown that human haemoglobin also manifests efficient catalytic activity towards CCl_4 [3]. In that study it was found, for the first time, that a significant amount of haem is irreversibly lost during the anaerobic metabolism of CCl_4 to carbon monoxide. It would appear that Hb haem plays a double, critical role in the reaction: that of the catalytic site for CCl_4 activation and of the target for the reactive metabolites formed. Such a dual role of haem had also been

Table 5. Protection by PBN against the halothane-dependent loss of human haemoglobin-derived haem

Incubation	nmol/mL	Haem (%)	% loss
Control	2.20 ± 0.08	(100)	0.0
+ halothane 5 mM	2.04 ± 0.04*	(93.0)	7.0
+ halothane 5 mM and PBN	2.12 ± 0.08§	(96.0)	4.0
+ halothane 10 mM	1.78 ± 0.03*	(81.0)	19.0
+ halothane 10 mM and PBN	2.02 ± 0.06†	(92.0)	8.0

* $P < 0.01$ vs control; § $P < 0.05$, and † $P < 0.001$, vs substrate alone; Student's *t*-test.

Incubations were made anaerobic as described in Materials and Methods and contained 2.25 ± 0.12 (mean ± SD) nmol Hb/mL. After preincubation, the reaction was started by addition of 1 mM sodium dithionite (control) or sodium dithionite and the indicated final concentrations of halothane. When indicated, 10 mM PBN was added before the substrate and the reducing agent. After a 15 min incubation, Hb-derived haem was determined by the haemochromogen–pyridine method. Values are the mean ± SD of five determinations.

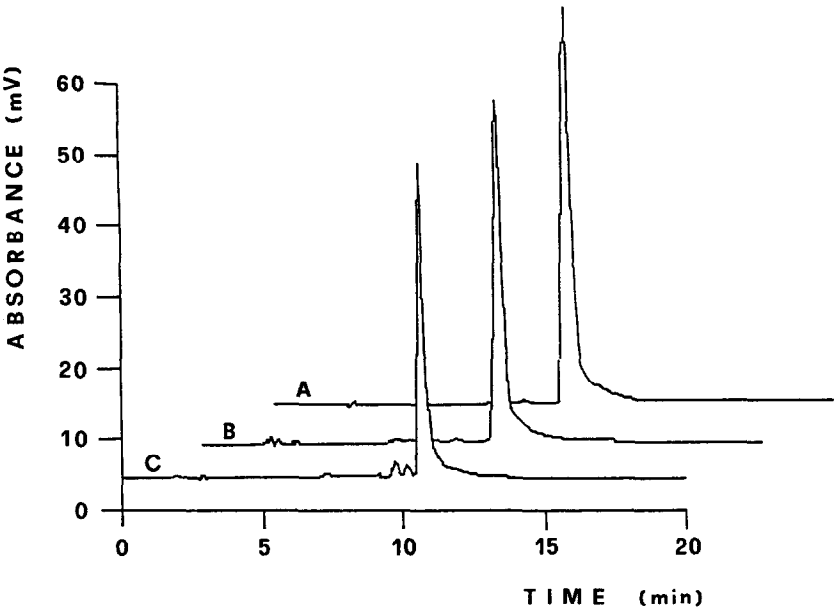

Fig. 5. HPLC chromatograms of haem and haem products after reductive incubation of haemoglobin with halothane. Incubations were made anaerobic as described in Materials and Methods and contained 37.30 ± 1.80 (mean ± SD) nmol Hb/mL. No addition was made to incubation A. After preincubation, the reaction was started by the addition to the incubation mixture of sodium dithionite alone (1 mM, final concentration) (B), or sodium dithionite and halothane (20 mM, final concentration) (C). After a 15 min incubation HPLC analysis was carried out as reported in Materials and Methods.

observed during the reductive activation of CCl_4 by rat and human liver P450 [7, 12].

The present results indicate that halothane, also a suicide substrate of rat and human P450 [11, 12], undergoes, like CCl_4, reductive activation by Hb. The available evidence indicates that electron transfer from the haem of Hb to the substrate is necessary for halothane activation by Hb, as shown by the following. First, no significant F^- production (Table 1) or Hb inactivation (Table 2) was found in the absence of sodium dithionite. Second, halothane metabolism was associated with conversion of Hb^{2+} to Hb^{3+} throughout the reaction and despite the presence of strong reducing conditions (Fig. 2). Third, two additional reducing systems selective for Hb were also able to support Hb haem loss (Table 4). Finally, a significant inhibition of Hb inactivation was observed in the presence of CO. The present results partly confirm and expand on early findings by Baker *et al.* [2] indicating that the protein portion of the enzyme is not required for reductive halothane dehalogenation. These authors showed that halothane releases inorganic fluoride when incubated anaerobically not only with NADPH- or sodium dithionite-reduced rat liver microsomes, but also with sodium dithionite-reduced boiled microsomes, heated haemoglobin and haemin. They found, however, that F^- production was very low when halothane was incubated with native non-heated Hb and suggested that heating may have facilitated the access of the substrate to the haem or, alternatively, released haem from Hb, thus making it available for the reaction. We also found in our study that native Hb was much less efficient than heated Hb in catalysing F^- formation (Table 1). The greater production of F^- observed in previous experiments [22], and confirmed in the present study (results not shown), when halothane was incubated with a water soluble haem preparation (MHA) in the presence of sodium dithionite, suggests that the latter hypothesis may be true.

Furthermore, the present data show that halothane metabolism may result, under anaerobic and reductive conditions, in the inactivation of the haemoprotein itself, as observed previously for CCl_4 [3]. The halothane-dependent loss of both Hb (Tables 2 and 3, Fig. 3) and Hb-derived haem (Tables 3 and 4) indicates that, during the reductive activation of the substrate, reactive metabolites are formed which attack the haemoprotein. The target of these reactive species is the haem prosthetic group and substrate activation is conditional upon interaction of the substrate with a free and reduced haem iron. Previous bubbling of the incubation mixture with the strong Hb^{2+} ligand carbon monoxide largely prevented Hb inactivation, as already found for the reaction in which microsomal P450 was the catalyst [11], indicating a similar reaction mechanism for the two haemoproteins.

The reactive metabolite of halothane responsible for Hb inactivation is not known. Several possible candidates may be considered. Upon addition of halothane to reduced anaerobic rat liver microsomes, a carbanion intermediate (CF_3CHCl^-) was proposed to form a complex with oxidized P450 haem to give a typical spectral change [20]. A free radical inter-

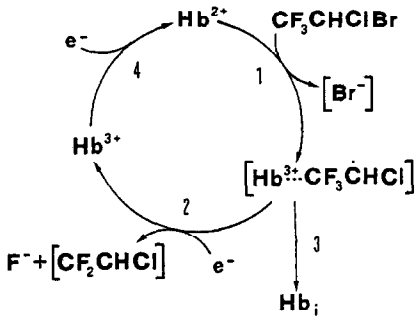

Fig. 6. Proposed mechanism for the reductive activation of halothane by human haemoglobin. (1) The reductive dehalogenation of the substrate forms a free radical metabolite which can either (2) undergo a second one electron reduction to form F^- and another stable metabolite, or (3) attack and inactivate haemoglobin (Hb_i). Finally, (4) Hb^{3+} can be reduced to enter a new catalytic cycle. The species in square brackets were not detected in the present study, but their presence in the reaction was derived from the literature.

mediate ($CF_3\dot{C}HCl$), detected both *in vitro* [23] and *in vivo* [24] as the product of halothane reductive debromination, has been indicated as the reactive species responsible for the attack on and the loss of P450 haem observed during the anaerobic reductive activation of halothane by liver microsomes [11]. The significant protection against the halothane-dependent loss of Hb-derived haem given by the spin trapping agent PBN (Table 5) indicates that with Hb a free radical intermediate may also be formed.

Hb inactivation is due to a modification of the haem moiety without destruction of its protoporphyrin IX tetrapyrrolic structure, as indicated by the absence of any significant loss of porphyrin fluorescence. The structure of the modified haem products formed during halothane metabolism remains to be investigated. The limited evidence available from the present HPLC data are consistent with the formation of at least two haem-derived compounds which retain a tetrapyrrolic ring but which are modified as to their ability to interact with pyridine or CO. The two substrate-dependent peaks observed in the chromatogram may be attributed to haem products in that they were eluted immediately before haem and showed a typical haem-like spectrum (Fig. 5). Work is in progress to isolate and characterize these Hb-derived halothane-dependent haem products.

The present results are similar to those obtained previously with CCl_4 under analogous reductive conditions [3], although halothane appears to be somewhat less efficient than CCl_4 in promoting the inactivating process. Approximately 50% Hb haem was lost when Hb was incubated with 5 mM CCl_4 [3] against only 12.4% when the same concentration of halothane was used (Table 3). However, the mechanism of the Hb-catalysed reaction is probably similar for the two substrates. A proposed scheme for the reductive activation of halothane by Hb is reported in Fig. 6. Reduced Hb interacts with halothane resulting in the reductive dehalogenation of the substrate and formation of a reactive free radical (1). Two alternative pathways are then possible: the free radical can either undergo a second electron reduction to form F^- and a stable metabolite (2), or attack the haem portion of the protein, leading to its irreversible inactivation (3). Finally, oxidized Hb can be reduced once again and enter a new redox cycle (4).

In conclusion, the present data indicate that under anaerobic and reducing conditions (i) human haemoglobin can activate halothane reductively, (ii) the classical haemoprotein spectrum with carbon monoxide is lost in a dose- and time-dependent manner, (iii) Hb^{2+} is oxidized to Hb^{3+} and (iv) haem, the prosthetic group of Hb, is irreversibly modified to products which are unable to give the typical pyridine–haemochromogen reaction but maintain an intact tetrapyrrolic structure. The toxicological significance of the present observation and its relevance for the aerobic *in vivo* situation remains to be elucidated. The reductive metabolism of halothane, as well as other polyhalogenated alkanes, by both animal and human liver P450 is thought to be responsible for various forms of toxicity caused by these chemicals. Based on the present data, however, a significant role of haemoglobin and, possibly, other haemoproteins in the biotransformation of these chemicals cannot be ruled out. The question might even be raised as to whether reductive activation of halothane and other halogenated alkanes to reactive metabolites by Hb may also occur *in vivo* and, possibly, contribute to their clinical or occupational toxicity.

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