The role of the liver in the production of free radicals during halothane anaesthesia in the rat

Quantification of *N*-tert-butyl- α -(4-nitrophenyl)nitron (PBN)-trapped adducts in bile from halothane as compared with carbon tetrachloride*

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Halothane or CCl₄ was co-administered with the spin trap *N*-tert-butyl- α -(4-nitrophenyl)nitron (PBN) to rats fitted with bile duct cannuli or to isolated perfused liver preparations. Rats maintained under halothane anaesthesia generated significant amounts of free radicals, and 5–9 nmol was excreted in bile over 1 h. No adducts were detected in urine or plasma. The hepatic origin of these free radicals was confirmed by studies on isolated perfused livers where the addition of halothane to the perfusate resulted in the biliary elimination of the same PBN-trapped radical adducts. Similarly, following CCl₄ administration, the same radical species were eliminated in bile in the whole animal and the perfused liver preparation. In the perfused liver, over 3 h the total biliary elimination of radicals derived from halothane or CCl₄ (administered at equimolar concentrations) was approximately the same (5–7 nmol); however, the elimination of halothane-derived radicals was more rapid over the first 1 h.

INTRODUCTION

The hepatotoxicity of many halogenated hydrocarbons has been attributed to the production of free radical intermediates (Slater, 1972; Reynolds & Moslen, 1980). However, until relatively recently, evidence for the participation of free radicals in cell damage has been circumstantial and largely based on the quantification of the products of free-radical-catalysed processes such as lipid peroxidation (Comporti *et al.*, 1965; Ghoshal & Recknagel, 1965; Slater, 1967; Brown, 1972) and the protection afforded by various free radical scavengers and detoxicating enzyme systems (Slater & Sawyer, 1971; Poli *et al.*, 1978; Cheeseman, 1982). The correlation would be much stronger if determined directly and related to the extent of cell damage. Unfortunately, the detection and hence quantification of free radicals in biological systems is inherently difficult because of their high reactivity and short half-lives, and early attempts to detect free radicals of CCl₄ were not successful (Keller, 1971; Calligaro & Vannini, 1975). However, problems of detection have been partially overcome using spin traps (Janzen, 1972, 1980), which allow the accumulation of stable radical adducts to concentrations ($> 10^{-8}$ M) which are detectable by conventional e.s.r. spectroscopy. Free radicals formed during the metabolism of the hepatotoxins halothane and CCl₄ have been successfully trapped with *N*-tert-butyl- α -(4-nitrophenyl)nitron (PBN) both *in vivo* and in several systems *in vitro* (Poyer *et al.*, 1978, 1980; Lai *et al.*, 1979; Albano *et al.*, 1982; Fujii *et al.*, 1984; McCay *et al.*, 1984). Studies *in vivo* have usually involved extraction of trapped adducts from liver tissue following administration of the toxin, and while those studies are important in identifying the type(s) of radical generated in the intact animal, attempts at correlating free radical production and damage have been limited

to the more versatile systems *in vitro* (Tomasi *et al.*, 1983; Cheeseman *et al.*, 1985). Although extrapolation of data obtained *in vitro* to the whole animal has been achieved, this is often fraught with difficulties; indeed, there are numerous examples where isolated cells do not mimic the whole organ, let alone the situation *in vivo* (Blom *et al.*, 1982; Miller *et al.*, 1983; Hughes, 1989; Powell *et al.*, 1989). Thus information on the amount and rate of production of free radicals in the whole animal or the intact liver is lacking. The aim of this study is to provide such information by exploiting the fact that PBN-trapped adducts formed in the liver undergo biliary elimination and are sufficiently concentrated therein to enable direct measurement (without extraction) by e.s.r. In this paper we provide quantification of PBN adducts eliminated in bile from the whole animal during halothane anaesthesia, evaluate the contribution of the liver using the isolated perfused rat system, and with the latter compare the amount of free radicals produced with that from an equimolar concentration of the hepatotoxin CCl₄.

MATERIALS AND METHODS

Materials

PBN, *N*-tert-butyl- α -(2-sulphophenyl)nitron, sodium salt (PBNS), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), 2-methyl-2-nitrosopropane dimer (MNP) and 3-doxyl-17 β -hydroxy-5 α -androstane (3DHA) were obtained from Aldrich, Gillingham, Dorset, U.K. Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane), CCl₄ (spectroscopic grade) and poly(ethylene glycol) (PEG) 200 were purchased from BDH Chemicals, Poole, Dorset, U.K. BSA was supplied by Boehringer Mannheim, Mannheim, Germany; heparin by Evans Medical Ltd., Greenford,

Abbreviations used: PBN, *N*-tert-butyl- α -(4-nitrophenyl)nitron; PEG, poly(ethylene glycol); PBNS, *N*-tert-butyl- α -(2-sulphophenyl)nitron; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; MNP, 2-methyl-2-nitrosopropane; 3DHA, 3-doxyl-17 β -hydroxy-5 α -androstane.

* This paper is dedicated to the late Professor Gillian M. Powell and Dr. Jeffrey C. Evans.

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Middlesex, U.K.; penicillin-G by Sigma Chemical Co., Poole, Dorset, U.K.; and Sagatal (sodium pentobarbitone) by May and Baker Ltd., Dagenham, Essex, U.K. All other reagents were of the highest grade available and were obtained from BDH Chemicals or Sigma Chemical Co. Bovine blood was supplied by a local abattoir (FMC, Bridgend, U.K.), and polyethylene tubing was from Portex, Hythe, Kent, U.K.

Animals

Adult male rats (200–300 g body wt.) of the Sprague–Dawley CD strain (bred within the Department of Biochemistry, U.W.C.C.) were used throughout. Animals were maintained on a standard laboratory diet (Pilsbury 41B; Pilsbury, Birmingham, U.K.) and allowed water *ad libitum*.

Experiments with anaesthetized rats

In experiments with halothane, rats were anaesthetized by inhalation of the test compound through a face mask. The vapour was produced by bubbling compressed air through a solution of halothane. Inhalation by this means was the sole route of administration of the test compound. A midline abdominal incision was made and the common bile duct was cannulated towards the liver with polyethylene tubing (internal diameter 0.28 mm, external diameter 0.61 mm). As soon as a continuous bile flow had been established, the body wall and skin were closed and sutured. PBN (40 mg, 0.24 mmol in 0.4 ml of PEG 200) was administered intravenously via the dorsal vein of the penis (designated zero time), and thereafter bile was collected in two samples (0–30 min and 30–60 min) into preweighed tubes. After 60 min, a urine sample (by means of a bladder puncture) and a blood sample (from the dorsal aorta) were taken. The blood sample (approx. 1 ml) was centrifuged at 1500 *g* for 5 min at 20 °C and the plasma was removed. Bile, plasma and urine samples were stored on ice (up to 1 h) until analysed by e.s.r. The liver was minced in an equal volume of water, then chloroform/methanol (2:1, v/v) was added (2 vol.) and the whole mixture was homogenized using a Silverson blender (Silverson Machines Ltd., Waterside, Chesham, Bucks., U.K.) The homogenate was centrifuged at 1500 *g* at 20 °C for 20 min, the aqueous and organic phases were separated and each was concentrated under a stream of nitrogen prior to analysis by e.s.r. In control experiments either halothane (by inhalation) or PBN (40 mg, 0.24 mmol in 0.4 ml of PEG 200, by intravenous injection) was administered. In the latter case, animals were anaesthetized with Sagatal (6 mg in 0.2 ml of 0.154 M-NaCl administered via the jugular cannula; polyethylene tubing: internal diameter 0.4 mm, external diameter 0.8 mm).

In experiments with CCl₄, rats were anaesthetized initially with diethyl ether, and in order to facilitate respiration during the experimental period (up to 3 h) the trachea was cannulated with polyethylene tubing (internal diameter 1.67 mm, external diameter 2.42 mm). The jugular vein was also cannulated (see above) and Sagatal was administered as required to maintain anaesthesia throughout the experimental period. Following a midline abdominal incision, the bile duct was cannulated and a small incision was made in the duodenum. A solution of PBN (40 mg, 0.24 mmol in 1 ml of corn oil) was injected into the lumen and the site of incision was immediately ligated to prevent leakage of the dosing solution into the peritoneal cavity. Bile was collected at 30 min intervals, and at the end of the experimental period urine and blood samples were taken and the liver was homogenized and extracted as described above. All samples were stored on ice prior to analysis by e.s.r. In control experiments, PBN (40 mg, 0.24 mmol) or CCl₄ (70 μ l, 1 mmol) in 1 ml of corn oil was injected into the lumen.

Perfused rat liver system

Livers were perfused in the recirculating system described by Powell *et al.* (1989). The perfusate consisted of washed bovine erythrocytes suspended in Krebs–Ringer bicarbonate buffer (Dawson, 1969), containing 3% (w/v) BSA, to give a packed cell volume of between 30 and 35%. Glucose (240 mg), heparin (500 units) and penicillin-G (3340 units) were added to each 120 ml of perfusate. Livers were isolated from donor rats under Sagatal anaesthesia (12 mg in 0.4 ml of 0.154 M-NaCl administered via the dorsal vein of the penis) using the surgical procedures outlined by Miller (1973). The common bile duct was cannulated and the hepatic portal vein and superior vena cava were cannulated with polyethylene tubing (internal diameter 1.67 mm, external diameter 2.42 mm). The liver was flushed free of blood with Krebs–Ringers bicarbonate buffer, at 37 °C, and was attached to the perfusion apparatus via the hepatic portal vein cannula. As soon as a satisfactory perfusate flow through the liver (30–50 ml/min) and bile flow (approx. 1 μ l/min per g of liver) had been established, solutions of PBN (80 mg, 0.48 mmol in 0.8 ml of PEG 200) and either halothane (108 μ l, 1 mmol in 0.5 ml of PEG 200) or CCl₄ (96 μ l, 1 mmol in 0.5 ml of PEG 200) were added to the perfusate. The distribution of PBN adducts between bile, perfusate supernatant and liver was determined over 3 h. The viability of the liver preparation over this time period under control conditions has been thoroughly investigated (Powell *et al.*, 1989). During perfusion, bile was collected at 30 or 60 min intervals, depending on the volume. Samples (2 ml) of the perfusate were removed at zero time (addition of test compound) and at 30 min intervals up to 3 h. The erythrocytes were removed by centrifugation and the supernatant from each sample was retained on ice (as were the bile samples) until analysed by e.s.r. At the end of the experimental period, the liver was retained and processed as described above. In addition, the remaining perfusate (approx. 85 ml) was centrifuged at 1500 *g* for 20 min at 20 °C and the supernatant was removed. This was freeze-dried and subsequently redissolved in a minimal volume of distilled water prior to e.s.r. analysis.

E.s.r. spectroscopy

Samples were placed in a quartz flat cell (Wilma WG 812; Wilma Glass W., Buena, NJ, U.S.A.) and spectra were recorded on a Varian E109 X-band spectrometer using 100 kHz field modulation. The spectrometer settings were, unless otherwise stated: power, 10 mW; modulation amplitude, 10⁻⁴ T; scan range, 0.1 T; scan time, 16 min; time constant, 1 s; ambient temperature. The spectral simulation was carried out using an OPUS 5 microprocessor and plotted on a Rowland digital plotter using in-house software. Spin-trapped radical adducts in bile were quantified by comparing the peak height of the *m* = -1 transit (outer line of the third doublet) with that obtained with known concentrations of 3DHA in bile run under identical conditions.

RESULTS

Biliary excretion of PBN adducts derived from halothane

The appearance of PBN adducts in bile (not subjected to any extraction procedures) collected over 1 h from a rat during halothane anaesthesia is shown in Fig. 1(a). At least two major species were detectable and were present in both the 0–30 and 30–60 min samples. The peak concentration of nitroxide adducts was in the 0–30 min sample, and this was 2–3-fold higher than that in the 30–60 min sample, despite the continuous inhalation of halothane (Table 1). A total of 5–9 nmol of PBN adducts was eliminated over the experimental period. In subsequent experiments using lower doses of PBN (10 mg, 0.06 mmol in

Fig. 1. Comparison of the e.s.r. spectra of bile collected from (a) a rat under halothane anaesthesia and following the intravenous administration of PBN (0.24 mmol) and (b) the isolated perfused rat liver following the addition of halothane (1 mmol) and PBN (0.48 mmol) to the perfusate

(a) i, 0–30 min; ii, 60–120 min. (b) i, 0–30 min; ii, 30–60 min; iii, 60–90 min; iv, 90–120 min. Gain settings were: (a) i, 2.5×10^3 ; ii, 6.3×10^4 ; (b) i–iv, 3.2×10^4 .

0.1 ml of PEG 200), a total of 1.6 nmol of the same adducts was eliminated in the 0–60 min period. This, together with the decline of the nitroxide signals in the bile with time, suggests that the amount of PBN was rate-limiting. Attempts to assign splitting constants to the major species present in bile proved unsuccessful, as the lack of hyperfine detail and the overlapping nature of the components prevented reliable simulation. Earlier spin-trapping studies with liver preparations exposed to halothane and PBN (*in vivo* and *in vitro*) reported by Poyer *et al.* (1981), Tomasi *et al.* (1983) and Fujii *et al.* (1984), however, showed a single species (A_N 1.44–1.50 mT, A_H 0.225–0.300 mT) to be present in organic extracts of the treated preparations, with a proposed structure of PBN/ $\text{Cl}\dot{\text{C}}\text{HCF}_3$ or PBN/ $\text{Br}\dot{\text{C}}\text{HCF}_3$. This component was subsequently and unequivocally identified using g.c./m.s. techniques as PBN/ $\text{Cl}\dot{\text{C}}\text{HCF}_3$ by Janzen *et al.* (1990). These authors in addition resolved, using $[\text{H}_{14}]$ -deuterated PBN to enhance hyperfine splitting, three further species in organic extracts of rat liver microsomal preparations following halothane and PBN treatment. These were two lipid radicals (a major

Table 1. Quantification of PBN adducts secreted into bile

PBN adducts were measured in the whole animal under halothane anaesthesia and following intravenous administration of PBN, and in the isolated perfused rat liver system (IPRL) following the addition of halothane (1 mmol) and PBN to the perfusate.

Model	PBN administered (mmol)	Bile sample time (min)	Concn. of PBN adducts in sample (μM)	Total PBN adducts in sample (nmol)
Whole animal ($n = 2$)	0.24	0–30	12.49, 19.00	3.14, 6.30
		30–60	6.63, 6.31	1.99, 2.31
		0–60	9.30, 12.66	5.13, 8.61
Whole animal	0.06	0–30	1.70	1.14
		30–60	0.87	0.42
		0–60	1.35	1.56
IPRL ($n = 2$)	0.48	0–30	7.44, 2.75	2.38, 1.10
		30–60	7.95, 5.33	2.43, 1.85
		60–90	5.93, 4.49	1.75, 1.44
		90–120	2.30, 4.64	0.38, 0.86
		0–120	6.39, 4.19	6.94, 5.25

Table 2. Quantification of PBN/ CCl_4 and PBN/ CO_2 adducts secreted into bile

Secretion by the isolated perfused rat liver ($n = 2$) was measured over 180 min following the addition of CCl_4 (1 mmol) and PBN (0.48 mmol) to the perfusate

Bile sample time (min)	Concn. of PBN/ CCl_4 (μM)	Total PBN/ CCl_4 (nmol)	Concn. of PBN/ CO_2 (μM)	Total PBN/ CO_2 in sample (nmol)	Total PBN adducts (nmol)
0–60	6.58, 12.09	1.28, 1.13	5.40, 15.72	1.05, 1.47	2.33, 2.60
60–120	4.90, 5.99	1.20, 1.62	5.47, 6.66	1.34, 1.80	2.54, 3.42
120–180	3.00, 1.26	0.50, 0.33	3.48, 1.38	0.58, 0.36	1.08, 0.69
0–180	4.91, 4.93	2.98, 3.08	4.90, 5.81	2.97, 3.63	5.95, 6.71

carbon-centred radical with partially assigned structure $\dot{\text{C}}\text{H}_2\text{R}$ and a minor oxygen-centred radical of the $\dot{\text{O}}\text{R}'$ type and a halocarbon double adduct of PBN (I-PBN-I), present in minor amounts. It is highly likely that some of these species are also present in bile in addition to PBN/ $\text{Cl}\dot{\text{C}}\text{HCF}_3$. There were no detectable signals in plasma or urine and only very weak signals were observed in the organic extracts of liver removed at the end of the 60 min experimental period from animals given the higher dose of PBN. In control experiments with animals given halothane or PBN alone, no nitroxide adducts could be detected in urine, bile or liver extracts. In experiments where PBN was replaced with other spin-trapping agents such as MNP (12.5 mg, 0.14 mmol in 0.5 ml of PEG 200) and DMPO (50 mg, 0.42 mmol in 0.5 ml of 0.154 M-KCl), also administered intravenously, no signals were detected in bile, plasma or urine, or in concentrated liver extracts. However, with PBNS (60 mg, 0.22 mmol in 0.6 ml of 0.154 M-NaCl, administered intravenously), which is a less lipid-soluble form of PBN, a weak and transient signal (A_N 1.575 mT, A_H 0.55 mT) was observed in bile from a rat under halothane anaesthesia. This signal was not present when PBNS was administered to animals under Sagatal anaesthesia.

In the perfused liver model in which halothane (1 mmol) and PBN (0.48 mmol) were added directly to the perfusate, the same two radical species as seen in the whole animal experiments were eliminated in bile. The peak concentration occurred between 30

Fig. 2. E.s.r. spectra of concentrated organic extract of rat liver following a 90 min perfusion with perfusate containing halothane (1 mmol) and PBN (0.48 mmol)

The gain was 2.5×10^4 .

Fig. 3. E.s.r. spectra of bile samples from the isolated perfused rat liver following the addition of halothane (1 mmol) and PBN (0.24 mmol) to the perfusate

(a) 0–30 min; (b) 30–60 min; (c) 60–90 min. The ascorbyl radical is denoted by •. Gain = 10×10^4 .

and 60 min (Fig. 1b), and over a 2 h perfusion period between 5 and 7 nmol of halothane-derived adducts were eliminated by this route (Table 1). Concentrated extracts of liver after a 2 h perfusion gave no signals, whereas extracts of liver taken after 1.5 h (Fig. 2) contained a single radical species (A_N 1.463 mT, A_H 0.225 mT) corresponding to that reported by Poyer & McCay (1981), Tomasi *et al.* (1983) and Fujii *et al.* (1984) for PBN/ ClCHCF_3 . Analysis of supernatants of perfusate samples

from these experiments revealed the ascorbyl radical; however, attempts to concentrate any other radical adducts present in trace amounts in the perfusate by freeze-drying resulted in the production of a radical species (A_N 1.550 mT, A_H 0.375 mT). The formation of this species is independent of halothane, since it is also observed after freeze-drying of residual perfusate supernatants from control experiments in which PBN (0.48 mmol) alone was added to the perfusate. The origin of this PBN adduct is unknown; however, e.s.r. analysis of concentrated PBN solutions (50 mg in 0.25 ml of ethanol; 1.05 M) gave no signals even at the highest sensitivities. This suggests either that hepatic metabolism of PBN is required and that freeze-drying merely concentrates the radicals present in the perfusate supernatant, or alternatively that free radicals are generated during the freeze-drying process. The latter phenomenon has been previously reported by Commoner *et al.* (1954). Following the administration of PBN alone to the perfused liver, no signals were detected in bile or liver extracts. In other control experiments in which halothane alone was administered, only the ascorbyl radical was detected in the bile and perfusate. The ascorbyl radical was also present in bile in test experiments in which halothane (1 mmol) and PBN at the lower dose of 40 mg (0.24 mmol in 0.4 ml of PEG 200) were administered to the perfusate. This radical was clearly evident in the 0–30 min sample (Fig. 3a), along with the same PBN adducts present at the higher dose of PBN (80 mg, 0.48 mmol, Fig. 1b) and increased in concentration with time, whereas the PBN adducts peaked in the 0–30 min sample and subsequently declined (Fig. 3).

Biliary excretion of PBN adducts derived from CCl_4

In the anaesthetized rat, receiving CCl_4 and PBN intraduodenally, the bile collected over 3 h showed one major PBN adduct, PBN/ CCl_3 (A_N 1.525 mT, A_H 0.335 mT), and small and variable amounts of a second species. Moreover, the intensity of signals was also extremely variable between individual experiments. Extraction of this major adduct from bile with 2 vol. of chloroform/methanol (2:1, v/v) eliminated the solvent effects of bile and decreased both the N and H splitting for PBN/ CCl_3 to 1.44 mT and 0.187 mT respectively. These values are identical with those reported by Knecht & Mason (1988) for this species in extracted bile and those obtained from concentrated organic liver extracts following perfusion with PBN and $^{13}\text{CCl}_4$ reported by Connor *et al.* (1986). Only the ascorbyl radical was observed in plasma and urine samples following PBN/ CCl_4 treatment. In control experiments in which CCl_4 alone was administered to an anaesthetized rat, the ascorbyl radical was present in bile (60–120 min sample) and urine. Following dosing with PBN, weak nitroxide signals were detected in bile over 3 h. Similar signals have previously been reported by Knecht & Mason (1988); their identity remains unknown, but they may be due to an impurity in the PBN, since the signals were not detected in earlier control experiments (see halothane section), where a different batch of PBN was used.

The addition of PBN (0.48 mmol) and CCl_4 (1 mmol) to the perfused rat liver resulted in the biliary excretion of two clearly defined nitroxide adducts (Fig. 4), PBN/ CCl_3 (A_N 1.52 mT, A_H 0.33 mT) and PBN/ CO_2 (A_N 1.60 mT, A_H 0.457 mT) which correspond to those eliminated in bile in the anaesthetized rat following PBN/ CCl_4 administration, as reported by Knecht & Mason (1988). The concentration of adducts obtained was highest over the first 2 h of perfusion and declined thereafter. A total of 6–7 nmol of adducts was excreted over 3 h. In concentrated liver extracts after this amount of time there were no detectable signals. In control experiments with CCl_4 alone there were no detectable signals in bile; organic extracts of liver or perfusate supernatant (prior to freeze-drying).

Fig. 4. Biliary elimination of PBN/ CCl_4 and PBN/ CO_2 in the isolated perfused rat liver over 180 min following the addition of CCl_4 (1 mmol) and PBN (0.48 mmol) to the perfusate

(a) 0–60 min; (b) 60–120 min; (c) 120–180 min. (d) Computer simulation of 120–180 min sample. The simulation was obtained for a mixture of PBN/ CCl_4 (▼) and PBN/ CO_2 (▽) in the ratio of 3:1, with line widths of 4×10^{-5} T and 2×10^{-5} T respectively. Ascorbyl radical is denoted by •. Gain for (a), (b) and (c) = 5×10^4 .

DISCUSSION

The production of free radicals derived from halothane and CCl_4 by reductive cytochrome *P*-450-dependent pathways is now well documented (Butler, 1961; Kubicek & Anders, 1981; Ahr *et al.*, 1982; Nastainczyk *et al.*, 1982; Cheeseman *et al.*, 1985). In unravelling these pathways and assessing their importance in hepatotoxicity, much attention has been focused on the enhanced production of PBN-trapped radicals *in vivo* and *in vitro* under hypoxic conditions and/or following pretreatment with phenobarbital (Fujii *et al.*, 1982; Tomasi *et al.*, 1983; Knecht & Mason, 1988). In the case of halothane, both of these manipulations are considered necessary because, in the rat, hepatic necrosis is only observed in phenobarbital-treated animals under hypoxic conditions (McClain *et al.*, 1979; Ross *et al.*, 1979; Plummer *et al.*, 1982; Van Dyke, 1982). However, the interrelationships between the degree of hypoxia, the induction of cytochrome *P*-450 and the metabolism of halogenated alkanes to free radicals and hepatotoxicity are complicated (Jee *et al.*, 1980; Shing *et al.*,

1982). Nevertheless, the present study demonstrates unequivocally that radical adducts are secreted continuously in bile from rats under halothane anaesthesia without prior induction with phenobarbital. Not only were these adducts detectable by direct examination of bile without prior extraction but, and this was particularly surprising, there was a higher concentration in bile than had hitherto been expected from previous tissue sampling techniques. Bile collected over 1 h from rats maintained under halothane anaesthesia contained between 5 and 9 nmol (or 0.5–0.9 nmol per g of liver), which is up to 2000 times greater than the 0.38 ± 0.15 pmol per g of tissue extracted from rat liver following 2 h of halothane anaesthesia in non-induced donor rats reported by Poyer *et al.* (1981), and between 4- and 8-fold higher than the levels reported by Fujii *et al.* (1984) in liver extracts from rats maintained under hypoxic conditions (10% oxygen) and following phenobarbital treatment. The hepatic origin of these unexpectedly high levels in bile was confirmed by studies with the isolated perfused rat liver in which between 5 and 7 nmol of adducts was excreted over 2 h following the addition of 1 mmol of halothane to the perfusate. Since the dose of halothane absorbed via the lungs is not known, the relative efficiency of free radical production in the two models cannot be assessed and the similar rates of adduct secretion in bile may be a coincidence. The dangers of quantifying free radicals in biological systems using spin traps have been alluded to by a number of authors (Evans, 1979; Janzen, 1980), and thus even the relatively high levels of trapped adducts recorded in the present study may be a minimum value. Indirect evidence for this is provided by the fact that the biliary signals are significantly lower when less PBN is used. Moreover, although there was no evidence for the passage of halothane-derived radicals into the perfusate in these studies, this is still a possibility (Connor *et al.*, 1986). Direct detection of small amounts of adducts is difficult due to dilution in the perfusate, and concentrating them by freeze-drying is not feasible, since this process itself produces significant amounts of halothane-independent PBN radical adducts.

The use of PBN to trap free radicals derived from CCl_4 *in vivo* and in the isolated perfused rat liver has been well documented (Lai *et al.*, 1979; Poyer *et al.*, 1980; Albano *et al.*, 1982; Connor *et al.*, 1986). After the addition of CCl_4 to the perfused rat liver, Connor *et al.* (1986) identified both PBN/ CCl_4 and PBN/ CO_2 adducts in extracts of liver and perfusate, although examination of bile was not reported. However, when PBN and CCl_4 were co-administered to phenobarbital-treated anaesthetized rats with bile duct fistuli, both the PBN/ CCl_4 and PBN/ CO_2 adducts, together with a possible lipid-derived radical adduct, were eliminated in the bile (Knecht & Mason, 1988). In the present study, although both adducts were detected in bile from the intact rat and the perfused liver, quantification of the two species was very variable in the anaesthetized rat model. This might be due in part to low and variable rates of adsorption from the gut lumen of the spin trap and/or CCl_4 . Indeed, work in our laboratory using the isolated perfused gut system has shown that the vehicle PEG 200 greatly decreases the transport of water-insoluble steroids such as [^{14}C]oestrone from the gut lumen into blood (Roy *et al.*, 1991). By contrast in the isolated perfused liver where the test compound and spin trap delivery is not a problem, the total amount of radical species in bile was consistently high (between 6 and 7 nmol in 3 h) and was comparable with the amounts derived from equimolar amounts of halothane.

In summary, the present work with halothane and CCl_4 highlights some of the advantages of the isolated perfused liver model for the quantification of hepatic free radical production. Firstly, compared with the whole animal, the perfused system allows for a more controlled delivery of both test compound and

spin trap. Secondly, compared with e.s.r. analysis of tissue extracts (following exposure *in vivo*), the continuous secretion of radical adducts into bile (and analysis therein) provides the time course of free radical production. Thirdly, and perhaps most importantly, considerably greater amounts of radicals are produced using this methodology than hitherto reported from liver extracts of rats following halothane anaesthesia. This ability to obtain more accurate data of amounts of radicals produced by the intact organ is clearly an important step forward in any attempt to relate the quantities of radicals produced to the nature and degree of liver damage incurred due to the action of known and suspected hepatotoxins.

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