Degradation of Cytochrome *P*-450 Haem by Carbon Tetrachloride and 2-Allyl-2-Isopropylacetamide in Rat Liver *in vivo* and *in vitro*

INVOLVEMENT OF NON-CARBON MONOXIDE-FORMING MECHANISMS

By Philip S. GUZELIAN and Robert W. SWISHER

Liver Study Unit, Medical College of Virginia, Richmond, VA 23298, U.S.A.

(Received 7 March 1979)

Degradation of intrinsic hepatic [^{14}C]haem was analysed as ^{14}CO formation in living rats and in hepatic microsomal fractions prepared from these animals 16 h after pulse-labelling with 5-amino[5- ^{14}C]laevulinic acid, a precursor that labels bridge carbons of haem in non-erythroid tissues. NADPH-catalysed peroxidation of microsomal lipids *in vitro* (measured as malondialdehyde) was accompanied by loss of cytochrome *P*-450 and microsome-associated [^{14}C]haem (largely cytochrome *P*-450 haem), but little ^{14}CO formation. No additional ^{14}CO was formed when carbon tetrachloride and 2-allyl-2-isopropylacetamide were added to stimulate lipid peroxidation and increase loss of cytochrome *P*-450 [^{14}C]haem. Because the latter effect persisted despite inhibition of lipid peroxidation with MnCl_2 or phenyl-*t*-butylnitron (a spin-trapping agent for free radicals), it was concluded that carbon tetrachloride, as reported for 2-allyl-2-isopropylacetamide, may promote loss of cytochrome *P*-450 haem through a non-CO-forming mechanism independent of lipid peroxidation. By comparison with breakdown of intrinsic haem, catabolism of [^{14}C]methaemalbumin by microsomal haem oxygenase *in vitro* produced equimolar quantities of ^{14}CO and bilirubin, although these catabolites reflected only 18% of the degraded [^{14}C]haem. This value was increased to 100% by addition of MnCl_2 , which suggests that lipid peroxidation may be involved in degradation of exogenous haem to products other than CO. Phenyl-*t*-butylnitron completely blocked haem oxygenase activity, which suggests that hydroxy free radicals may represent a species of active oxygen used by this enzyme system. After administration of carbon tetrachloride or 2-allyl-2-isopropylacetamide to labelled rats, hepatic [^{14}C]haem was decreased and haem oxygenase activity was unchanged; however, ^{14}CO excretion was either unchanged (carbon tetrachloride) or decreased (2-allyl-2-isopropylacetamide). These changes were unaffected by cycloheximide pretreatment. From the lack of parallel losses of cytochrome *P*-450 [^{14}C]haem and ^{14}CO excretion, one may infer that an important fraction of hepatic [^{14}C]haem in normal rats is degraded by endogenous pathways not involving CO. We conclude that carbon tetrachloride and 2-allyl-2-isopropylacetamide accelerate catabolism of cytochrome *P*-450 haem through mechanisms that do not yield CO as an end product, and that are insensitive to cycloheximide and independent of haem oxygenase activity.

It is generally accepted that physiological degradation of haem in the liver involves scission of the porphyrin ring specifically at the α -methene bridge, with conversion of the bridge carbon to CO, and release of iron and biliverdin-IX α . This process is mediated by haem oxygenase, a microsomal enzyme that utilizes NADPH and molecular O_2 in converting haem *in vitro* to equimolar quantities of CO and biliverdin-IX α (Tenhunen *et al.*, 1966). CO formation has been used as a convenient means for measuring haem degradation because this end product is not reutilized *in vivo* and is assumed to be specific for

haem degradation and to maintain a strict stoichiometric relationship to catabolized haem (Ludwig *et al.*, 1957). Further studies have demonstrated that, in rats given the haem precursor 5-amino[5- ^{14}C]laevulinic acid, which labels hepatic haem (in the bridge carbon atoms) to the exclusion of haemoglobin haem, changes in excretion of ^{14}CO from the intact animal quantitatively reflect changes in the degradation of hepatic haem, primarily microsomal cytochrome *P*-450 (Bissell & Hammaker, 1976).

We have utilized this system of ^{14}CO collection from living rats to investigate mechanisms by which hepatotoxins perturb the steady-state concentration of cytochrome *P*-450. Some toxins appear to ac-

Abbreviation used: haem, ferriprotoporphyrin-IX α .

celerate the degradation of hepatic cytochrome *P*-450 haem by stimulating 'physiological' (CO-forming) haem catabolism. Thus, when administered to animals injected with 5-amino[5- 14 C]laevulinic acid, endotoxin (Bissell & Hammaker, 1976), bromobenzene (Guzelian & Bissell, 1976) and cobalt (Guzelian & Barwick, 1979) all stimulate 14 CO release, decrease hepatic cytochrome *P*-450 [14 C]-haem content and induce hepatic haem oxygenase activity. These changes are preventable by pretreatment of rats with cycloheximide (Guzelian & Barwick, 1979). In preliminary studies *in vivo* with two other well-known hepatotoxins, we found that carbon tetrachloride and 2-allyl-2-isopropylacetamide decreased hepatic [14 C]haem content without an accompanying stimulation of 14 CO excretion (Guzelian & Bissell, 1976), which suggests the involvement of a non-CO-forming mechanism for haem breakdown. Although others have reported previously that 2-allyl-2-isopropylacetamide or carbon tetrachloride degrades cytochrome *P*-450 haem *in vitro* to 'abnormal' products other than bilirubin, CO formation was not examined in these studies (Levin *et al.*, 1972; De Matteis, 1971). The 'abnormal' haem catabolites formed by carbon tetrachloride are clearly not identical with those formed by 2-allyl-2-isopropylacetamide, since the latter appear as 'green pigments' (Schwartz & Ikeda, 1955; De Matteis, 1973), the product of direct attack on cytochrome *P*-450 haem by 2-allyl-2-isopropylacetamide or its metabolites (Ortiz de Montellano *et al.*, 1978; Correia *et al.*, 1979). Until recently, carbon tetrachloride was believed to act indirectly by stimulating formation of lipid peroxides (reviewed by Recknagel, 1967), because this process is associated with loss of cytochrome *P*-450 haem and quantitative formation of CO (Estabrook *et al.*, 1971; De Matteis & Sparks, 1973; Levin *et al.*, 1973; Schacter *et al.*, 1973; Iba *et al.*, 1977). However, the CO formed in hepatic microsomal preparations undergoing lipid peroxidation does not appear to originate from radio-labelled endogenous haem (De Matteis *et al.*, 1977). In the present report, we have analysed haem degradation, both in microsomal preparations and in the living rat, and find that 2-allyl-2-isopropylacetamide and carbon tetrachloride promote breakdown of haem by non-CO-forming pathways that do not depend on lipid peroxidation.

Materials and Methods

Materials

Male Sprague-Dawley rats, weighing 150–200g, were purchased from Flow Laboratories (Dublin, VA, U.S.A.). The animals were housed individually in wire-bottomed cages and maintained on standard laboratory chow diet. Spectrally pure carbon

tetrachloride was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); phenyl-*t*-butylnitron was from Eastman Chemical Co. (Rochester, NY, U.S.A.); hopcalite catalyst for oxidation of CO was from Mine Safety Appliances Co. (Pittsburgh, PA, U.S.A.). 2-Allyl-2-isopropylacetamide was a gift from Hoffman-La Roche (Nutley, NJ, U.S.A.). 5-Amino[5- 14 C]laevulinic acid was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.); other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Methods

Preparation of hepatic microsomes (microsomal fractions). Animals were starved overnight and then killed by decapitation. Livers were perfused with iced 0.1M-potassium phosphate buffer (pH 7.4) and then excised and homogenized in the same buffer (20%, w/v). The homogenate was centrifuged at 600g for 10min and then at 9000g for 20min. The supernatant was removed and recentrifuged at 105000g for 60min. The supernatant was discarded and the microsomal pellet was resuspended in 0.1M-potassium phosphate buffer (pH 7.4). Microsomes containing [14 C]haem were prepared by the same procedure, except that the rats received 6 μ Ci of 5-amino[5- 14 C]laevulinic acid in 0.1 ml of 0.9% NaCl by tail-vein injection 16h before being killed. For preparation of post-mitochondrial supernatant used in studies of [14 C]methaemalbumin degradation *in vitro*, rats were injected subcutaneously with 80mg of CoCl₂/kg 15h before being killed, to induce haem oxygenase activity (Maines & Kappas, 1974), and the 9000g supernatant was prepared as described above.

Measurement of endogenous microsomal [14 C]haem degradation and 14 CO production *in vitro*. The reaction mixture consisted of 0.1mM-NADPH, 20–30mg of microsomal protein and sufficient 0.1M-potassium phosphate buffer to give a final volume of 10ml. The reaction was carried out in a stoppered 50ml Erlenmeyer flask incubated in the dark at 37°C with shaking for 15min, and was terminated by addition of menadione (1 μ M, final concentration). 14 CO was collected throughout the incubation by drawing a continuous flow of air over the surface of the reaction solution by vacuum suction. The collected air was passed first through Mallcosorb (Mallinckrodt Chemical Co., Jersey City, NJ, U.S.A.) to remove CO₂, and then through heated hopcalite to convert 14 CO to 14 CO₂ (Landaw *et al.*, 1970). Finally, the 14 CO₂ was trapped in 4ml of ethanolamine/2-methoxyethanol (1:2, v/v), and the radioactivity measured in a Beckman LS-9000 liquid-scintillation spectrometer. Recovery of 14 CO was tested for each combination of additives to the reaction mixture (see Table 1) by passing a known amount of 14 CO

(formed by adding [^{14}C]formic acid to heated H_2SO_4) through the reaction solution containing unlabelled microsomes. In all cases, recovery of ^{14}C was greater than 90%, and the present data have been corrected to 100% efficiency. Addition of $\text{K}_3\text{Fe}(\text{CN})_6$ did not improve ^{14}CO recovery (White, 1968). The final concentrations of other agents added in some experiments were: 4.7 mM-carbon tetrachloride, 1 mM- MnCl_2 , 4 mM-2-allyl-2-isopropylacetamide, 75 mM-phenyl-t-butyl nitron. Added NADPH gave linear and reproducible release of ^{14}CO for reactions carried out for up to 20 min. Because in experiments with 2-allyl-2-isopropylacetamide 30 min was required to yield significant degradation of [^{14}C]haem, an NADPH-generating system was substituted for NADPH. This system consisted of (final concentrations) 0.4 mM-NADPH, 9.0 mM-glucose 6-phosphate, 0.6 mM- MgCl_2 , 1.2 mM-nicotinamide and 3 units of glucose 6-phosphate dehydrogenase/ml. When ^{14}CO collections had been completed, portions of the reaction mixture were taken for measurements of lipid peroxides as described below, or were centrifuged at 105 000g for 60 min to prepare microsomes for measurement of cytochrome P-450, total radioactivity and [^{14}C]haem. By subtraction of the latter two values, 'non-haem' radioactivity was calculated. For each experiment, a control incubation was carried out with the appropriate additions minus either NADPH or NADP^+ .

Lipid peroxidation. This was estimated by measuring thiobarbituric acid-reacting substances as described previously (Bidlack *et al.*, 1973): 3 ml of the reaction mixture was mixed with 0.3 ml of 20% (w/v) trichloroacetic acid and the precipitate was centrifuged at 7000g for 10 min. Then 1 ml of the supernatant was combined with 1 ml of 0.75% thiobarbituric acid solution and, after the mixture had been heated in boiling water for 20 min, the absorbance at 532 nm was measured in a Beckman model 24 spectrophotometer. The results were expressed as total nmol of malondialdehyde formed/min per incubation flask, assuming a millimolar absorption coefficient of 156 litre $\cdot$ mmol $^{-1}$ $\cdot$ cm $^{-1}$.

Degradation of exogenous [^{14}C]haem in vitro. [^{14}C]Haem was extracted from [^{14}C]haemoglobin, prepared biosynthetically with 5-amino[5- ^{14}C]laevulinic acid as precursor (Goldstein *et al.*, 1968), and was recrystallized to constant specific radioactivity (Shemin *et al.*, 1950). [^{14}C]Methaemalbumin solution was prepared by dissolving 2 mg of [^{14}C]haem in 0.8 ml of 0.2 M-NaOH containing 1.9 mg of Tris and 35.5 mg of NaCl. Then 1.6 ml of 1% human serum albumin was added and the pH was adjusted to 7.4. The reaction mixture containing (final concentration) 26.7 μM -[^{14}C]methaemalbumin, 0.1 mM-NADPH, 10 mg of 9000g supernatant protein and sufficient 0.1 M-potassium phosphate buffer (pH 7.4)

to give a final volume of 10 ml was incubated for 15 min. ^{14}CO was collected, and [^{14}C]haem disappearance was measured as described above for endogenous microsomal haem. To measure bilirubin formation, the same reaction mixture was incubated at 37°C in the sample cuvette of an Aminco split-beam spectrophotometer (American Instrument Co., Silver Spring, MD, U.S.A.), while the reference cuvette contained reaction mixture with buffer substituted for NADPH. The A_{468} was measured for 15 min and the concentration of bilirubin was calculated by using 60 litre $\cdot$ mmol $^{-1}$ $\cdot$ cm $^{-1}$ as the millimolar absorption coefficient (Bissell *et al.*, 1972).

Measurement of ^{14}CO excretion and haem degradation in vivo. Animals were labelled by intravenous administration of 6 μCi of 5-amino[5- ^{14}C]laevulinic acid/200 g body wt. and, after 16 h when degradation of the labelled haem in the rapidly turning over haem fractions was completed (Bissell & Hammaker, 1976), each of a pair of animals was placed in tandem metabolic cages connected to parallel CO -collecting systems. When control measurements of ^{14}CO were completed (in some instances several hours were required to achieve stable ^{14}CO -excretion rates), one rat was injected with either carbon tetrachloride or 2-allyl-2-isopropylacetamide, the other with 0.9% NaCl. Collections of ^{14}CO were continued for 4 h. For reasons of economy and convenience, and to minimize the effects of inter-animal variability in initial labelling of hepatic haem, the effects of carbon tetrachloride and 2-allyl-2-isopropylacetamide on hepatic [^{14}C]haem content were examined in a single study rather than by a series of individual experiments in combination with ^{14}CO collections. Animals were injected with 5-amino[5- ^{14}C]laevulinic acid and, after starvation overnight for 16 h, the groups received intraperitoneal injections of 2-allyl-2-isopropylacetamide (300 mg/kg), carbon tetrachloride (1 ml/kg in corn oil, 50%, v/v) or 0.9% NaCl. Injections of corn oil had no effect on ^{14}CO excretion in control experiments. After 4 h the animals were killed, and the livers were perfused and excised. Three pairs of livers from each group were individually homogenized. Cytochrome P-450, total radioactivity and [^{14}C]haem contents were measured in each of the homogenates and in microsomes prepared from each homogenate. Microsomal haem oxygenase activity was assayed by using 105 000g supernatant from normal rat liver as the source of biliverdin reductase (Bissell *et al.*, 1972).

Other procedures. Cytochrome P-450 was measured as the dithionite-reduced plus CO minus reduced difference spectrum in microsomes (Omura & Sato, 1964), and in homogenate with CO substituted for dithionite in the reference cuvette (Raj & Estabrook, 1970). Haem was extracted and crystallized by using

Table 1. *Lipid peroxidation, [¹⁴C]haem degradation and ¹⁴CO formation in hepatic microsomes in vitro*
Incubations were made with microsomes prepared from rats pretreated with 5-amino[5-¹⁴C]laevulinic acid as described under 'Methods'. Results are given as means $\pm$ s.d. for three to five experiments. The final concentrations of MnCl₂ and phenyl-t-butyl-nitron were 1 mM and 75 mM respectively. Expected value assumes the molar ratio of haem breakdown and ¹⁴CO production equals 1; it was calculated as:

Additions to the reaction mixture	d.p.m. of $^{14}\text{CO} \times 8 \times 100$				^{14}CO production (% of expected value)
	Ad.p.m. of [^{14}C]haem (control – experimental)		Microsomes		
	Lipid peroxidation (nmol of malondialdehyde/ min per flask)	Cytochrome P-450 (% of control)	[^{14}C]Haem (% of control)		
Controls	<1.0	100	100		<0.5
NADPH	19.2 $\pm$ 5.1*	43.3 $\pm$ 10.1*	51.3 $\pm$ 23.0*		2.6 $\pm$ 3.7*
NADPH + MnCl ₂	1.7 $\pm$ 0.33*	92.1 $\pm$ 5.6	96.5 $\pm$ 4.9		<0.5
NADPH + phenyl-t-butyl/nitron	<1.0	96.2 $\pm$ 7.2	98.2 $\pm$ 2.8		<0.5
NADPH + carbon tetrachloride	30.6 $\pm$ 8.9*	23.3 $\pm$ 10.4*	37.1 $\pm$ 15.0*		4.1 $\pm$ 3.2*
NADPH + carbon tetrachloride + MnCl ₂	3.9 $\pm$ 1.6*	40.5 $\pm$ 6.3*	77.5 $\pm$ 10.6*		2.5 $\pm$ 2.0*
NADPH + carbon tetrachloride + phenyl-t-butyl/nitron	<1.0	56.5 $\pm$ 7.0*	73.6 $\pm$ 6.3*		<0.5
NADPH + 2-allyl-2-isopropylacetamide	11.9 $\pm$ 2.4*	48.0 $\pm$ 16.0*	68.8 $\pm$ 12.9*		3.1 $\pm$ 1.9*
NADPH + 2-allyl-2-isopropylacetamide + MnCl ₂	<1.0	68.1 $\pm$ 4.3*	78.0 $\pm$ 3.2*		6.1 $\pm$ 2.1*
NADPH + 2-allyl-2-isopropylacetamide + phenyl-t-butyl/nitron	<1.0	83.2 $\pm$ 12.5*	78.3 $\pm$ 10.2*		<0.5

* Value significantly different from control ($P < 0.05$).

rat erythrocytes as carriers, as described previously (Labbe & Nishida, 1957). Haem was measured as the pyridine haemochrome (Paul *et al.*, 1953), and protein by the technique of Schacterle & Pollack (1973) with crystalline bovine albumin as standard.

Results and Discussion

Degradation of [¹⁴C]haem in vitro (see Table 1)

We studied the radiolabelled haem in hepatic microsomes prepared from rats 16h after pulse administration of 5-amino[5-¹⁴C]laevulinic acid. Under this protocol, microsome-associated [¹⁴C]-haem is largely cytochrome P-450 haem (Bissell & Hammaker, 1976). Lipids extracted from labelled microsomes (Bligh & Dyer, 1959) contained less than 0.01 % of the total radioactivity. Control incubations of microsomes suspended in buffer alone or with the additives listed in Table 1 (except NADPH) gave little or no loss of cytochrome P-450 or [¹⁴C]haem content and no detectable lipid peroxidation. Upon addition of NADPH to the reaction mixture, the concentration of cytochrome P-450 and of microsome-associated [¹⁴C]haem markedly decreased, whereas lipid peroxidation was stimulated. When inhibitors that decrease (MnCl₂) or abolish (phenyl-t-butyl nitron) lipid peroxidation were added, the disappearance of cytochrome P-450 and [¹⁴C]haem was prevented (Table 1). The fact that phenyl-t-butyl nitron, a spin-trapping agent that forms stable adducts with reactive free radicals (Poyer *et al.*, 1978), abolished lipid peroxidation is in line with the postulate that lipid peroxidation may be initiated by hydroxy free radicals (Fong *et al.*, 1973) rather than by excited-state oxygen species such as singlet oxygen or the superoxide anion radical (Pederson & Aust, 1975). Despite loss of approx. 50 % of the [¹⁴C]haem in incubations containing NADPH alone, the production of ¹⁴CO was less than 3 % of the amount that would be expected if each mol of degraded [¹⁴C]haem released 1 mol of ¹⁴CO (Table 1). These results support suggestions that the CO formed in microsomes undergoing NADPH-dependent lipid peroxidation does not arise from degraded microsome-associated haem (De Matteis *et al.*, 1977), but rather from catabolized microsome lipids (Wolff & Bidlack, 1976). The recovery of total radioactivity in microsomes incubated with NADPH or with any of the other reactants listed in Table 1 was always greater than 90 %. No attempt was made to characterize the microsome-associated non-haem radioactive products. It is possible that these products represent altered forms of haem that are not measured by spectral assays for cytochrome P-450 or by radiometric assays for haem, even though the haem may not truly be 'destroyed' or 'degraded' when defined as cleavage of the tetrapyrrole ring with release of bridge carbon atoms. An alternative possibility is that

the non-haem radioactivity formed in microsomes incubated with NADPH constitute water-soluble dipyrroles (propentdyopents) (Guengerich, 1978). If this finding is correct, then the lack of formation of haem-derived CO associated with NADPH-catalysed lipid peroxidation implies that the oxidative cleavage of the tetrapyrrole ring or the subsequent metabolism of bridge carbon atoms (or both) must differ from the process mediated by haem oxygenase.

It is believed that carbon tetrachloride exerts its toxic effects on cytochrome P-450 indirectly, by stimulating formation of lipid peroxides [reviewed by Slater (1966) and by Recknagel & Glende (1973)], which are catalytically decomposed by cytochrome P-450, destroying its haem moiety in the process (Hrycay & O'Brien, 1971). We found that addition of carbon tetrachloride to incubations containing NADPH magnified the stimulation of lipid peroxidation and the decrease in cytochrome P-450 and [¹⁴C]haem observed with NADPH alone (Table 1). The disappearance of [¹⁴C]haem seen with carbon tetrachloride plus NADPH resembled that associated with NADPH-catalysed lipid peroxidation in producing only small amounts of ¹⁴CO (Table 1). Attempts have been made to dissociate carbon tetrachloride-stimulated lipid peroxidation from loss of cytochrome P-450 by using EDTA, but these were unsuccessful (Reiner *et al.*, 1972). In preliminary experiments, we found, as have others (Masuda & Murano, 1977), that over a wide range of concentrations, EDTA inhibited no more than 80 % of tetrachloride-stimulated formation of malondialdehyde. In testing MnCl₂, we found that the augmented lipid peroxidation with carbon tetrachloride was decreased to a value similar to that formed by NADPH plus MnCl₂ (3.9 versus 1.7 nmol of malondialdehyde per flask), whereas the decreases in cytochrome P-450 and [¹⁴C]haem were only partially blocked (Table 1). This suggests that loss of cytochrome P-450 haem with carbon tetrachloride may not require lipid peroxidation. However, since the formation of lipid peroxides and loss of cytochrome P-450 need not be directly proportional, it was important to confirm this result by completely abolishing lipid peroxidation. When this was done by adding phenyl-t-butyl nitron to incubations containing NADPH plus carbon tetrachloride, cytochrome P-450 and [¹⁴C]haem decreased to 56.5 and 73.6 % respectively of the control values, even though lipid peroxidation was undetected (Table 1). Little ¹⁴CO was formed under these conditions (Table 1), and therefore loss of [¹⁴C]haem with carbon tetrachloride independent of lipid peroxidation appears also to involve a non-CO-forming mechanism. Since both cytochrome P-450 and [¹⁴C]haem decreased, it is unlikely that these results are due to interference by carbon tetrachloride with spectral measurements of cytochrome P-450 (Ota *et*

Table 2. *Effects of inhibitors of lipid peroxidation on degradation of [14 C]haem by haem oxygenase*

The source of haem oxygenase was 18000g supernatant prepared from liver homogenates of rats pretreated for 24h with CoCl_2 . Incubations of liver supernatant, [14 C]methaemalbumin, and the indicated additions were made for 15 min as described under 'Methods'. The concentration of MnCl_2 was 1 mM, and of phenyl-t-butyl-nitron was 75 mM. Loss of [14 C]haem was calculated from the difference between the initial and final values for radioactivity in extracted and crystallized haem and the specific radioactivity of the added [14 C]methaemalbumin. The latter value was also used to calculate ^{14}CO production with the assumption that 1 mol of haem is degraded to 1 mol of ^{14}CO . Bilirubin formation was measured colorimetrically (see under 'Methods'). The results for single findings in two experiments A and B are given. Abbreviation: NT, not tested.

Additions to reactions	Loss of [14 C]haem (nmol per flask)		^{14}CO production (nmol/15 min)		Bilirubin formation (nmol/15 min per flask)	
	A	B	A	B	A	B
None (control)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
NADPH	21.6	44.0	3.7	8.1	NT	8.5
NADPH+ MnCl_2	4.8	12.7	4.28	11.37	5.1	11.2
NADPH+phenyl-t-butyl-nitron	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

al., 1975), although this phenomenon may explain the larger decrease in cytochrome *P*-450 as compared with [14 C]haem (Tables 1 and 3). Because lack of CO formation does not distinguish between haem disappearance associated with NADPH-catalysed lipid peroxidation and that caused by carbon tetrachloride independent of lipid peroxidation, additional research is needed to determine if these processes form different non-haem radioactive products. One may speculate that, since metabolism of carbon tetrachloride to trichloromethyl radical is known to occur at or near cytochrome *P*-450 in the presence of phenyl-t-butyl-nitron (Poyer *et al.*, 1978), those radicals that do not migrate sufficiently far to be trapped by phenyl-t-butyl-nitron may directly attack the haem of cytochrome *P*-450. An analogous mechanism explaining the loss of cytochrome *P*-450 haem with 2-allyl-2-isopropylacetamide is the demonstration of a stable adduct formed between an 2-allyl-2-isopropylacetamide metabolite and cytochrome *P*-450 haem (Ortiz de Montellano *et al.*, 1978; Correia *et al.*, 1979).

Incubation of microsomes with NADPH and 2-allyl-2-isopropylacetamide gave results analogous to those with carbon tetrachloride (see Table 1). 2-Allyl-2-isopropylacetamide decreased cytochrome *P*-450 and [14 C]haem and stimulated lipid peroxidation. Nevertheless, when lipid peroxidation in the presence of 2-allyl-2-isopropylacetamide was blocked with either MnCl_2 or phenyl-t-butyl-nitron the losses in cytochrome *P*-450 and [14 C]haem were only partially prevented. Disappearance of [14 C]haem with 2-allyl-2-isopropylacetamide in the absence of lipid peroxidation was associated with only small amounts of ^{14}CO formation, a result consistent with similar experiments (De Matteis *et al.*, 1977).

Since loss of haem under all the conditions studied in Table 1 involved only non-CO-forming mech-

anisms, we performed a positive control experiment examining degradation of exogenous haem. When liver postmitochondrial supernatant as a source of haem oxygenase activity was incubated aerobically with NADPH and [14 C]methaemalbumin, equal amounts of ^{14}CO and bilirubin were produced (Table 2), as expected (Tenhunen *et al.*, 1966). However, ^{14}CO production accounted for only 18% of the [14 C]haem disappearance during the incubation, a discrepancy that implies that a substantial portion of haem loss occurred through a non-CO-forming mechanism. We were unable to determine directly whether this loss of haem was due to lipid peroxidation in these reactions, because methaemalbumin interfered with the colorimetric assay for malondialdehyde. Nevertheless, when MnCl_2 was added to the haem oxygenase reaction mixture in sufficient concentration (Table 1) to block lipid peroxidation, equimolar amounts of ^{14}CO and bilirubin were formed, stoichiometrically reflecting nearly 100% of the [14 C]haem lost (Table 2). These results suggesting that differences in rates of endogenous microsomal lipid peroxidation and hence in the degradation of haem by non-CO-forming mechanisms offer a plausible explanation for the disturbing lack of agreement among several laboratories in the percentage of degraded haem appearing as CO and bilirubin in haem oxygenase reactions. Reported values include less than 20% (present study), 77% (Tenhunen *et al.*, 1966) or 100% (De Matteis *et al.*, 1977). We were unable to confirm this hypothesis with phenyl-t-butyl-nitron as an inhibitor of lipid peroxidation, because, unlike MnCl_2 , phenyl-t-butyl-nitron was found to also be a potent inhibitor of haem oxygenase activity (Table 1). Although the nature of the active oxygen species in the haem oxygenase mechanism remains uncertain and controversial (Guengerich, 1978), our finding provides new evidence supporting the postulated

involvement of the hydroxy free radical (Masters & Schacter, 1976).

Haem degradation in vivo

In rats given 5-amino[5- ^{14}C]laevulinic acid 16h before control collections of ^{14}CO , administration of 2-allyl-2-isopropylacetamide (300mg/kg) produced a marked decrease in ^{14}CO excretion which was detectable within 30min (Fig. 1). In five experiments, the average decrease in cumulative excretion of ^{14}CO after 4h was $25.8 \pm 1.6\%$ ($\pm$ S.D.), when compared with ^{14}CO collected from the control animal. The decrease in ^{14}CO excretion was proportional to the dose of 2-allyl-2-isopropylacetamide in the range 200–400mg/kg (results not shown). 2-Allyl-2-isopropylacetamide treatment decreased hepatic [^{14}C]haem and increased non-haem radioactivity (Table 3), although the magnitude of the decrease in [^{14}C]haem (46%) was approx. twice that of the decrease in ^{14}CO excretion. Administration of carbon tetrachloride (1 ml/kg) produced no change in

Fig. 1. Effect of carbon tetrachloride or 2-allyl-2-isopropylacetamide on ^{14}CO excretion in vivo

In each experiment, pairs of rats were given 5-amino[5- ^{14}C]laevulinic acid 16h before the start of control collections of ^{14}CO (see under 'Methods'). At time 0 (indicated by arrow), one rat received 0.9% NaCl (control, ●), the other, carbon tetrachloride (a, ○) or 2-allyl-2-isopropylacetamide (b, ○), and hourly ^{14}CO collections were continued.

the rate of ^{14}CO excretion, an effect consistently observed in five experiments (Fig. 1). Nevertheless, this dose of carbon tetrachloride significantly decreased (17%) the hepatic [^{14}C]haem content after 4h and increased non-haem radioactivity (Table 3). Because haem oxygenase activity in microsomes (see Table 3) prepared from rats given 0.9% NaCl, carbon tetrachloride or 2-allyl-2-isopropylacetamide were in the same range (results not shown), the lack of correspondence between ^{14}CO formation and [^{14}C]haem loss with carbon tetrachloride or 2-allyl-2-isopropylacetamide is not likely to be due to impaired enzymic capacity for 'physiological' haem oxidation in the liver. The effects of 2-allyl-2-isopropylacetamide and carbon tetrachloride were selective for the liver in that no significant changes were seen in kidney [^{14}C]haem content (Table 3). Higher doses of carbon tetrachloride (up to 3ml/kg) have not yet been systematically examined because these doses produced variable responses, consisting usually of an initial decrease in ^{14}CO excretion for 1–2h followed by a sharp rise in ^{14}CO excretion thereafter. Pre-treatment of labelled animals with cycloheximide (4mg/kg, intraperitoneally) 1h before administration of 2-allyl-2-isopropylacetamide or carbon tetrachloride had no effect on the subsequent loss of hepatic haem in homogenate or in microsomes (results not shown). Our results in the living rat are consistent with experiments *in vitro* in establishing that the rate of catabolism of endogenous hepatic haem is accelerated by 2-allyl-2-isopropylacetamide and carbon tetrachloride through pathways that do not involve CO. In contrast with the 'physiological' (CO-forming) pathway recruited by some hepatotoxins for stimulating haem breakdown (Guzelian & Bissell, 1976; Guzelian & Barwick, 1979), the alternative pathway(s) used by carbon tetrachloride or 2-allyl-2-isopropylacetamide may be characterized as being non-CO-forming, insensitive to cycloheximide and not associated with induction of haem oxygenase activity.

A previous study with 2-allyl-2-isopropylacetamide-treated rats suggested that hepatic degradation of exogenous haem or of newly synthesized intrinsic haem involved a non-CO-forming mechanism (Landaw *et al.*, 1970). With the present experimental design, it is possible to quantitatively compare ^{14}CO formation and degradation of [^{14}C]haem in stable haemoproteins because pulse-labelled animals are examined in the absence of continued synthesis of 'free' radioactive haem. Our results reveal that, after treatment with carbon tetrachloride or 2-allyl-2-isopropylacetamide, loss of [^{14}C]haem was significantly larger than the decline in ^{14}CO production. This finding is inconsistent with the postulate that degradation of cytochrome P-450 haem in the basal steady state is exclusively controlled by haem oxygenase (Bakken *et al.*, 1972; Maines, 1976). If the

Table 3. Effects of carbon tetrachloride and 2-allyl-2-isopropylacetamide on hepatic and renal [^{14}C]haem in rats given 5-amino[5- ^{14}C]laevulinic acid. According to the protocol described under 'Methods', rats with hepatic and renal haem prelabelled with 5-amino[5- ^{14}C]laevulinic acid were treated with 2-allyl-2-isopropylacetamide (300 mg/kg), carbon tetrachloride (1 ml/kg) or 0.9% NaCl and, 4 h later, the livers and kidneys were removed and homogenized, and microsomes were prepared. The indicated measurements were made on material pooled from two rats, and each value is the average of three measurements. Values in parentheses indicate percentage change relative to control.

Tissue fraction	Total [^{14}C]haem		Total protein (mg/liver)	Total cytochrome P-450 (nmol/liver)
	($10^{-6} \times \text{d.p.m. per liver}$)	(% of total radioactivity)		
Liver homogenate				
Control	2.456 $\pm$ 0.215	51	1197 $\pm$ 229	513 $\pm$ 156
Carbon tetrachloride	2.045 $\pm$ 0.244 (-17%)*	38	1185 $\pm$ 62	323 $\pm$ 84 (-37%)*
2-Allyl-2-isopropylacetamide	1.335 $\pm$ 0.012 (-46%)**	30	1002 $\pm$ 239	436 $\pm$ 97 (-15%)
Liver microsomes				
Control	1.185 $\pm$ 0.108	50	361 $\pm$ 68	265 $\pm$ 54
Carbon tetrachloride	0.943 $\pm$ 0.097 (-20%)*	40	422 $\pm$ 22	196 $\pm$ 30 (-26%)*
2-Allyl-2-isopropylacetamide	0.715 $\pm$ 0.072 (-40%)*	35	394 $\pm$ 28	197 $\pm$ 4.8 (-26%)*
Kidney homogenate				
Control	0.693 $\pm$ 0.032	23	301 $\pm$ 124	
Carbon tetrachloride	0.775 $\pm$ 0.036 (+12%)	22	373 $\pm$ 39	
2-Allyl-2-isopropylacetamide	0.794 $\pm$ 0.133 (+15%)	21	411 $\pm$ 33	

* Value significantly different from control ($P < 0.05$).

** Value significantly different from control ($P < 0.001$).

latter concept were correct, then the haem loss mediated by carbon tetrachloride or 2-allyl-2-isopropylacetamide should be reflected by a corresponding loss of substrate for haem oxygenase and, as a consequence, an equal decrease in ^{14}CO production. This argument makes the assumption based on studies *in vitro* (Table 1) that loss of [^{14}C]haem in carbon tetrachloride or 2-allyl-2-isopropylacetamide-treated rats yields little or no ^{14}CO . An alternative possibility is that in control animals a portion of the labelled hepatic haem, which can be identified indirectly by its sensitivity to attack by carbon tetrachloride or 2-allyl-2-isopropylacetamide, is destined for degradation by endogenous non-CO-forming mechanisms. It should be noted that because renal [^{14}C]haem may contribute a minor but significant portion of basal ^{14}CO excretion in these experiments (P. S. Guzelian, unpublished work), the size of this postulated haem fraction cannot be measured accurately. Prompted by this novel hypothesis, we are directly comparing [^{14}C]haem degradation and ^{14}CO excretion *in vivo* in nephrectomized rats and have found that as much as 50% of the labelled haem associated with microsomal cytochromes in normal rat liver appears to be degraded by endogenous non-CO-forming processes (D. M. Bissell & P. S. Guzelian, unpublished work). Therefore, although degradation of hepatic haem by 2-allyl-2-isopropylacetamide may proceed exclusively by an abnormal toxic mechanism (Unsold & De Matteis, 1976; Ortiz de Montellano *et al.*, 1978; Correia *et al.*, 1979), carbon tetrachloride may exert its effects not only through toxic mechanisms, but also by stimulating non-CO-forming pathways native to the liver.

We thank Ms. Joyce Barwick for technical assistance with some of these experiments, and Marcia Tetlak for expert typing of the manuscript. P. S. G. is the recipient of a Clinical Investigator Award in Gastroenterology from the NIH (no. 5K08AM00128). This work was supported by a grant from the NIH (no. 1P01AM18976).

References

- Bakken, A. F., Thaler, M. M. & Schmid, R. (1972) *J. Clin. Invest.* **51**, 530-536
- Bidlack, W. R., Okita, R. T. & Hochstein, P. (1973) *Biochem. Biophys. Res. Commun.* **53**, 458-465
- Bissell, D. M. & Hammaker, L. E. (1976) *Arch. Biochem. Biophys.* **176**, 91-102
- Bissell, D. M., Hammaker, L. & Schmid, R. (1972) *J. Cell Biol.* **54**, 107-119
- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911-917
- Correia, M. A., Farrell, G. C., Schmid, R., Ortiz de Montellano, P. R., Yost, G. S. & Mico, B. A. (1979) *J. Biol. Chem.* **254**, 15-17
- De Matteis, F. (1971) *Biochem. J.* **124**, 767-777
- De Matteis, F. (1973) *Drug Metab. Dispos.* **1**, 256-258

- De Matteis, F. & Sparks, R. G. (1973) *FEBS Lett.* **29**, 141–144
- De Matteis, F., Gibbs, A. H. & Unseld, A. (1977) *Biochem. J.* **168**, 417–422
- Estabrook, R. W., Franklin, N., Baron, J., Shigematsu, A. & Hildebrandt, A. (1971) in *Drugs and Cell Regulation* (Mihich, E., ed.), pp. 227–254, Academic Press, New York and London
- Fong, K. L., McCay, P. B., Poyer, J. L., Keele, B. B. & Mirsa, H. (1973) *J. Biol. Chem.* **248**, 7792–7797
- Goldstein, G. W., Hammaker, L. & Schmid, R. (1968) *Blood* **31**, 388–395
- Guengerich, F. P. (1978) *Biochemistry* **17**, 3633–3639
- Guzelian, P. S. & Barwick, J. (1979) *Biochem. J.* **180**, 621–630
- Guzelian, P. S. & Bissell, D. M. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1393
- Hrycay, E. G. & O'Brien, P. G. (1971) *Arch. Biochem. Biophys.* **147**, 14–27
- Iba, M. M., Soyha, L. F. & Schulman, M. P. (1977) *Mol. Pharmacol.* **13**, 1092–1104
- Labbe, R. F. & Nishida, G. (1957) *Biochim. Biophys. Acta* **26**, 437
- Landaw, S. A., Callahan, W. E., Jr. & Schmid, R. (1970) *J. Clin. Invest.* **49**, 914–925
- Levin, W., Jacobson, M. & Kuntzman, R. (1972) *Arch. Biochem. Biophys.* **148**, 262–269
- Levin, W., Lu, A. Y. H., Jacobson, M., Kuntzman, R., Poyer, J. L. & McCay, P. B. (1973) *Arch. Biochem. Biophys.* **158**, 842–852
- Ludwig, G. D., Blakemore, W. S. & Drabkin, D. L. (1957) *J. Clin. Invest.* **36**, 912
- Maines, M. D. (1976) in *Microsomes and Drug Oxidations* (Ullrich, V., Hildebrandt, A., Roots, I., Estabrook, R. & Conney, A., eds.), pp. 543–550, Pergamon Press, Oxford
- Maines, M. D. & Kappas, A. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4293–4297
- Masters, B. S. S. & Schacter, B. A. (1976) *Ann. Clin. Res.* **8**, 18–27
- Masuda, Y. & Murano, T. (1977) *Biochem. Pharmacol.* **26**, 2275–2282
- Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378
- Ortiz de Montellano, P. R., Mico, B. A. & Yost, G. S. (1978) *Biochem. Biophys. Res. Commun.* **83**, 132–137
- Ota, M., Sato, N., Vemura, H. & Obara, K. (1975) *Chem.-Biol. Interact.* **11**, 265–276
- Paul, K.-G., Theorell, H. & Akeson, A. (1953) *Acta Chem. Scand.* **7**, 1284–1287
- Pederson, T. C. & Aust, S. D. (1975) *Biochim. Biophys. Acta* **385**, 232–241
- Poyer, J. L., Floyd, R. A., McCay, P. B., Janzen, E. G. & Davis, E. R. (1978) *Biochim. Biophys. Acta* **539**, 402–409
- Raj, P. & Estabrook, E. W. (1970) *Pharmacologist* **12**, 261
- Recknagel, R. O. (1967) *Pharmacol. Rev.* **19**, 145–208
- Recknagel, R. O. & Glende, E. A., Jr. (1973) *Crit. Rev. Toxicol.* **2**, 263–297
- Reiner, O., Athanassopoulos, S., Hellmer, K. H., Murray, R. E. & Uehle, K. E. (1972) *Arch. Toxikol.* **29**, 219–233
- Schacter, B. A., Marver, H. S. & Meyer, U. A. (1973) *Drug. Metab. Dispos.* **1**, 286–290
- Schacterle, G. R. & Pollack, R. L. (1973) *Anal. Biochem.* **51**, 654–655
- Schwartz, S. & Ikeda, K. (1955) in *Ciba Found. Symp. Porphyrin Biosynthesis and Metabolism* (Wolstenholme, G. E. W. & Miller, E. C. P., eds.), pp. 209–226, Churchill, London
- Shemin, D., London, I. M. & Rittenberg, D. (1950) *J. Biol. Chem.* **183**, 757–765
- Slater, T. F. (1966) *Nature (London)* **209**, 36–40
- Tenhunen, R., Marver, H. S. & Schmid, R. (1966) *J. Biol. Chem.* **244**, 6388–6394
- Unseld, A. & De Matteis, F. (1976) in *Porphyrins in Human Diseases* (Doss, M., ed.), pp. 71–75, S. Karger, Basel
- White, P. (1968) *J. Clin. Invest.* **47**, 102
- Wolff, D. G. & Bidlack, W. R. (1976) *Biochem. Biophys. Res. Commun.* **73**, 850–867