Spin-trapping studies on the free-radical products formed by metabolic activation of carbon tetrachloride in rat liver microsomal fractions isolated hepatocytes and *in vivo* in the rat

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1. The metabolic activation of carbon tetrachloride to free-radical intermediates is an important step in the sequence of disturbances leading to the acute liver injury produced by this toxic agent. Electron-spin-resonance (e.s.r.) spin-trapping techniques were used to characterize the free-radical species involved. 2. Spin trapping was applied to the activation of carbon tetrachloride by liver microsomal fractions in the presence of NADPH, and by isolated intact rat hepatocytes. The results obtained with the spin trap *N*-benzylidene-2-methylpropylamine *N*-oxide ('phenyl *t*-butyl nitron') (PBN) and [¹³C]carbon tetrachloride provide unequivocal evidence for the formation and trapping of the trichloromethyl free radical in these systems. 3. With the spin trap 2-methyl-2-nitrosopropane, however, the major free-radical species trapped are unsaturated lipid radicals produced by the initiating reaction of lipid peroxidation. 4. Although pulse radiolysis and other evidence support the very rapid formation of the trichloromethyl peroxy radical from the trichloromethyl radical and oxygen, no clear evidence for the trapping of the peroxy radical was obtainable. 5. The effects of a number of free-radical scavengers and metabolic inhibitors on the formation of the PBN–trichloromethyl radical adduct were studied, as were the influences of changing the concentration of PBN and incubation time. 6. High concentrations of the spin traps used were found to have significant effects on cytochrome *P*-450-mediated reactions; this requires caution in interpreting results of experiments done in the presence of PBN at concentrations greater than 50 mM.

Free-radical intermediates with short biological half-lives have been demonstrated to be of importance in many examples of tissue injury that require the metabolic activation of a primary toxic agent (for reviews see Slater, 1972; Pryor, 1976–1980; Mason, 1979). Because such types of free radical generally have very short lifetimes and, moreover, since their rate of production is usually rather low, the concentrations of these free radicals are frequently below the level of detection by direct e.s.r. spectroscopy. For example, attempts to detect the formation of a free-radical product resulting from

the metabolic activation of CCl₄ in rat liver were not successful (Keller *et al.*, 1971; Slater, 1972; Burdino *et al.*, 1973; Calligaro & Vannini, 1975).

The spin-trapping technique has been developed for e.s.r. studies on transient free radicals in chemical and biological systems (Perkins *et al.*, 1970; Janzen, 1971, 1980). In that technique the free radical interacts with a spin trap possessing either a nitroso or nitron functional group to form a comparatively long-lived nitroxyl radical adduct. The application of the spin-trap procedure to biological problems is still at a developmental stage, and most experience so far has been with liver microsomal suspensions or purified enzyme systems (for review and references see McCay *et al.*, 1980). A few studies have been made, however, with spin traps and intact cells: for

Abbreviations used: PBN, *N*-benzylidene-2-methylpropylamine *N*-oxide ('phenyl *t*-butyl nitron'); MNP, 2-methyl-2-nitrosopropane.

instance with polymorphonuclear leucocytes (Rosen & Klebanoff, 1979; Green *et al.*, 1979) and with isolated hepatocytes (Tomasi *et al.*, 1980).

Carbon tetrachloride is a much studied hepatotoxin (Recknagel, 1967; Slater, 1972; Recknagel *et al.*, 1977; Dianzani, 1982) that produces centrilobular necrosis and fatty degeneration of the liver. CCl_4 is actively metabolized by isolated hepatocytes, and this metabolic activation is associated with an increased lipid peroxidation (Poli *et al.*, 1978) and with decreases in protein synthesis and in lipoprotein secretion (Poli *et al.*, 1979). The activated intermediate resulting from the metabolism of CCl_4 by the NADPH-cytochrome *P*-450 electron-transport chain in liver endoplasmic reticulum is generally considered to be the trichloromethyl free radical $\text{CCl}_3\cdot$ (Slater, 1972; Recknagel *et al.*, 1977; McCay *et al.*, 1980). Previous attempts to trap the $\text{CCl}_3\cdot$ free radical with 2-methyl-2-nitrosopropane in liver microsomal suspensions (Ingall *et al.*, 1978) were not successful, but more recently Poyer *et al.* (1980) and Tomasi *et al.* (1980) have reported the successful trapping of $\text{CCl}_3\cdot$ in liver microsomal fractions (referred to below simply as 'microsomes') with the spin trap *N*-benzylidene-2-methylpropylamine *N*-oxide (PBN). Preliminary evidence for the trapping of $\text{CCl}_3\cdot$ by PBN in isolated hepatocytes (Tomasi *et al.*, 1980), has also been given, and successful trapping of CCl_4 -derived free-radical species has been obtained under conditions *in vivo* (Lai *et al.*, 1979).

In the present study we have extended previous investigations (Tomasi *et al.*, 1980) on the interaction of $\text{CCl}_3\cdot$ with PBN in isolated hepatocytes, and revise the interpretation of earlier work with 2-methyl-2-nitrosopropane (MNP) (Ingall *et al.*, 1978) in the light of our additional information. Our objectives have been to positively identify the occurrence of $\text{CCl}_3\cdot$ free radicals in isolated hepatocytes, and in rats *in vivo*, during exposure to CCl_4 , and to investigate the effects of various competing free-radical scavengers on the formation of the PBN- $\text{CCl}_3\cdot$ adduct.

Materials and methods

N-Benzylidene-2-methylpropylamine *N*-oxide and 2-methyl-2-nitrosopropane were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; $^{13}\text{CCl}_4$ was purchased from New England Nuclear Corp. (Dreieich, West Germany). Collagenase (type I), lipoxygenase (type I), arachidonic acid, linolenic acid and linoleic acid were supplied by Sigma Chemical Co. (Poole, Dorset, U.K.). Promethazine, compound SKF 525A (2-diethylaminoethyl 3,3-diphenylpropylacetate) and (+)-catechin were kindly provided by May and Baker (Dagenham, Essex,

U.K.), Smith, Kline and French (Welwyn Garden City, Herts., U.K.) and Zyma S.p. (Nyon, Switzerland) respectively.

Adult male albino rats (body wt. approx. 200 g) were maintained on a standard laboratory diet (Labsure Animal Food, Christopher Hill Group, Poole, Dorset, U.K.) and water *ad libitum*. Rats were killed by cervical dislocation, and microsomes were prepared as previously described (Slater & Sawyer, 1971); the microsomal pellets were re-suspended in 0.15 M-KCl immediately before use to give a concentration equivalent to 1 g original wet wt. of liver/ml of suspension. Incubations of microsomal suspensions at 37°C in an NADPH-generating stock solution, and the addition of CCl_4 , were done as described by Slater & Sawyer (1971).

The demethylation of aminopyrene by liver microsomes in an NADPH-generating buffered solution was measured by the method given by Orrenius (1965), with the use of the formaldehyde determination method of Nash (1953). Cytochrome *P*-450 difference spectra were obtained as described by Schenkman *et al.* (1967), with a Carey 219 spectrophotometer.

Rat hepatocyte suspensions were prepared by the method of Poli *et al.* (1979). Samples (2 ml) of the suspension, containing 10^7 cells/ml, were incubated at 37°C in stoppered Erlenmeyer flasks (capacity 50 ml) that had central wells in which was placed 10 μl of CCl_4 when required. Under these conditions, the concentration of CCl_4 in the hepatocyte suspension at the end of the 10 min incubation was approx. 140 μM (Poli *et al.*, 1979).

The uptake of Trypan Blue by hepatocytes, the release of lactate dehydrogenase and the production of malonaldehyde by hepatocytes were measured as previously described (Poli *et al.*, 1979).

N-Benzylidene-2-methylpropylamine *N*-oxide (PBN) was dissolved in 0.15 M-KCl for experiments with microsomes, and in hepatocyte incubation medium for hepatocyte experiments. The final concentrations used are given in the text below.

In some experiments the microsomal and hepatocyte suspensions were shaken after incubation with PBN with a half-volume of chloroform/methanol (2:1, v/v); the chloroform layer was used for e.s.r. analysis. For the latter purpose, a Varian E.3 spectrometer was used, fitted with a variable-temperature cavity. In general, the spectrometer settings were: microwave power, 12.5 mW; modulation amplitude, 1 G (0.1 mT); scan range, 100 G (10 mT); temperature, -70°C for extracted samples, room temperature for the unextracted samples.

2-Methyl-2-nitrosopropane (MNP) was dissolved in acetone immediately before use, and kept protected from the light. Incubations with microsomes were performed in a flat cell at room temperature, or for 10 min at 37°C if extracted as described above.

Lipoxygenase experiments were performed as follows: a solution of lipoxygenase (EC 1.13.11.12) (3 mg/ml; 510 000 units/ml, where 1 unit $\equiv$ 0.12 μ mol of linoleic acid oxidized/min) in KHCO₃/CO₂ buffer, pH 9, was mixed with an equal volume of a suspension of linoleic acid, linolenic acid or arachidonic acid (3 mM) in water plus 0.5% sodium deoxycholate, flushed briefly with O₂, and then 20 mM-MNP solution was added before the e.s.r. recording.

Experiments *in vivo* were performed by injecting unlabelled CCl₄, or unlabelled CCl₄ 30% enriched with ¹³CCl₄, together with PBN intraperitoneally into rats; the rats were killed after 15 min, and samples of the livers were homogenized in 0.15 M-KCl. Lipid extractions were performed by the procedure of Folch *et al.* (1957). The PBN adduct solution for e.s.r. analysis was concentrated by partial removal of the chloroform solvent under an atmosphere of N₂. In these experiments the amount of CCl₄ used was 0.1 ml in 0.9 ml corn oil; the ¹³CCl₄ was 50% ¹³C : ¹²C; the amount of PBN injected was 17 mg in 0.9 ml of corn oil.

The covalent binding of ¹⁴CCl₄ to microsomal protein was determined by the procedure of Cheeseman (1981).

Results

Experiments with 2-methyl-2-nitrosopropane (MNP)

Incubation of rat liver microsomes with NADPH, CCl₄ and MNP at 37°C extracted in chloroform/methanol (2:1) resulted in the formation of a nitroxyl radical adduct with a characteristic e.s.r. triplet hyperfine splitting of 1.5 mT (Fig. 1a). With short incubation times no significant e.s.r. signal was observed in the absence of CCl₄ (Fig. 1b) or without NADPH. Replacement of CCl₄ with CHCl₃ also resulted in an absence of the strong triplet signal (Fig. 1c), but CBrCl₃ gave a more powerful signal response than found with CCl₄. The formation of the nitroxyl adduct thus appeared to be dependent on the presence of CCl₄ and NADPH; the intensity of the triplet signal varied with the incubation time up to a maximum intensity at approx. 5 min. Rat liver microsomes incubated in a flat cell placed in the e.s.r. cavity in the presence of NADPH, CCl₄ and MNP for longer than 5 min resulted in a composite spectrum, as shown in Fig. 2(b). The set of four lines is due to the t-butyl hydronitroxide, presumably derived by microsomal reduction of MNP (Kalyanaraman *et al.*, 1979a); the remaining absorptions are formed by the overlapping of different radicals.

When ¹³CCl₄ was used in place of unlabelled CCl₄ in similar experiments to those described above, there was no detectable splitting of the main triplet signal, as would be expected to occur if the adduct

was formed from the following reaction:

In consequence, the trapped species can be considered to be a secondary species derived from the interaction of CCl₃· with its local environment. Possibilities for such a secondary species are (Ingall *et al.*, 1978) the trichloromethylperoxyl radical (CCl₃O₂·) or a lipid-derived carbon-centred free radical of a polyunsaturated fatty acid (PUFA·). Since the CCl₃O₂· species is very reactive (Packer *et al.*, 1978, 1980), then it probably would not be effectively trapped by MNP in the concentrations that can be used in these experiments. Moreover, the adduct formed from CCl₃O₂· and MNP would be expected to show a hyperfine splitting *a_N* of approx. 2.7 mT rather than the much smaller value experimentally observed (Fig. 1a). We conclude that the likely species trapped in the results described in Figs. 1(a) and 2(b) are lipid-derived radicals.

Evidence consistent with this conclusion was derived by the incubation of linoleic acid, linolenic acid and arachidonic acid with lipoxygenase and MNP (Figs. 2c, 2d and 2e). The hyperfine splitting constants were *a_N* 1.53 mT and *a_H* 0.21 mT for the radical adduct derived from linoleic acid, *a_N* 1.57 mT and *a_H* 0.20 mT for that derived from linolenic acid, and *a_N* 1.44 mT and *a_H* 0.275 mT for that derived from arachidonic acid. These values are consistent with carbon-centred radicals.

The overlapping of different MNP-fatty acid radical adducts could give rise to a resulting spectrum close to that shown in Fig. 2(b).

The extraction of the same samples in chloroform/methanol (2:1) causes a broadening of the lines and consequently the disappearance of the hydrogen hyperfine splitting; the resulting spectra are similar to those shown in Fig. 1(a). Therefore we assign the spectra arising from microsomes incubated in the presence of NADPH, CCl₄ and MNP, either extracted in organic solvent or not, to a lipid derivative radical adduct formed by the interaction of CCl₄ with polyunsaturated fatty acids.

The MNP adduct formed by incubating CCl₄, NADPH, microsomes and MNP at 37°C was unstable; attempts to form the MNP-CCl₃· adduct by direct radiolysis of CCl₄ with MNP (Ingall *et al.*, 1978) were complicated by the oxidative breakdown of the primary adducts to yield an acyl product as described by Janzen (1980). Direct photochemical formation of CCl₃· in the presence of MNP in quartz tubes in the e.s.r. spectrometer cavity were successful in demonstrating adduct formation (Symons *et al.*, 1982). However, the adduct signal decayed very rapidly when the irradiation source

Fig. 1. E.s.r. spectra from rat liver microsomes and MNP

Rat liver microsomes were incubated for 5 min at 37°C, with an NADPH-generating system (see the Materials and methods section), 8 mM-MNP in acetone and 1 μ l of CCl₄ (a), 1 μ l of CHCl₃ (b) or without NADP (c), and then extracted in an equivalent volume of chloroform/methanol (2:1, v/v). The chloroform layer was used for the e.s.r. analysis. For experimental details see the text.

was turned off, again indicating the instability of the MNP adduct with CCl₃•. For the reasons outlined in the above discussion, we turned to another spin trap, PBN, for subsequent experiments.

Experiments with *N*-benzylidene-2-methylpropylamine *N*-oxide (PBN)

The e.s.r. spectrum obtained on incubating isolated rat hepatocytes with CCl₄ and PBN is illustrated in Fig. 3(b); the effect produced by omitting CCl₄ is shown in Fig. 3(a), and the result produced by using ¹³CCl₄ is shown in Fig. 3(c). The spectrum given in Fig. 3(b) shows a strong triplet of doublets with hyperfine splitting constants a_N 1.4 mT and a_H 0.175 mT. The introduction of ¹³C produced a splitting of the spectrum lines (a_N 0.97 mT), as shown in Fig. 3(c), indicating that the ¹³C nucleus is located β to the nitroxyl radical. The hyperfine splitting effect due to ¹³C was proportional to the atom% of ¹³C relative to ¹²C in the CCl₄ added to the system. The above results were all obtained by extracting the incubation mixtures with chloroform/methanol and using the chloroform layer for analysis, but essentially the same results were also seen with unextracted hepatocyte suspension placed directly in the e.s.r. spectrometer cavity. This result suggests that the PBN-CCl₃• adduct is mainly localized in the lipid environment of the membrane, since we have noticed a very little solvent effect on the hyperfine splittings.

The hyperfine features of the triplet of doublets shown in Fig. 3(b) are consistent with results obtained by high-energy radiolysis of CCl₄ in the presence of PBN (Symons *et al.*, 1982), and allow the assignment of the adduct as that formed by addition of CCl₃• to PBN. It is important to note that spectra identical with those discussed above for isolated hepatocytes have also been obtained by using rat liver microsomes with PBN (50 mM) and by injecting PBN (100 mg/kg body wt.) intraperitoneally into rats after they had been dosed with CCl₄ (Figs. 4b and 4c). Our results are in essential agreement with those obtained by Poyer *et al.* (1980), who used microsomes and also studied the situation *in vivo*.

The effects of varying the incubation time at 37°C and the concentration of hepatocytes in the suspension of the e.s.r. signal in the presence of PBN are shown in Fig. 5. The signal intensity increases with increasing time of incubation up to about 15 min, but thereafter there is no significant change. The e.s.r. response clearly increases with the number of cells in the suspension up to the maximum concentration studied here (20 $\times$ 10⁶/ml; Fig. 5).

Varying the concentrations of PBN in the hepatocyte/CCl₄ mixture produced the results shown in Fig. 6; there was an increase in signal intensity up to a PBN concentration of about 35 mM, but thereafter the intensity decreased. The reason for the decrease seen at high PBN concentration

Fig. 2. MNP adduct spectra from rat liver microsomes and peroxidized fatty acids

Rat liver microsomes were incubated as described for Fig. 1 in the presence (b) or in the absence (a) of CCl_4 . E.s.r. spectra were recorded without extraction and by incubating the microsome suspension in a flat cell placed directly in the spectrometer cavity. The spectra of MNP adducts with peroxidized linoleic acid (c), linolenic acid (d) and arachidonic acid (e), obtained by incubating for 5 min at room temperature a 3 mM fatty acids suspension in 0.5% sodium deoxycholate with soya-bean lipoxygenase (3 mg/ml) and 20 mM-MNP, are shown for comparison; the spectra were recorded without extraction, as described above. Abbreviation: DPPH, diphenylpicrylhydrazyl.

probably derives from an interaction of PBN with the cytochrome *P*-450 system and its role as a free-radical scavenger (see below). Higher concentrations of PBN than 35 mM are not suitable for use with isolated hepatocytes for the reason discussed above, although even up to 50 mM-PBN and with 30 min incubation at 37°C there were no

Fig. 3. E.s.r spectra of $\text{PBN-CCl}_3\cdot$ adduct in isolated hepatocytes

Isolated liver cells (10^7 cells/ml) were incubated for 10 min at 37°C with 25 mM-PBN alone (a) or with PBN and 10 μl of CCl_4 placed in the central well of the incubation flasks (b). Spectrum (c) was obtained as described for spectrum (b) except that 10 μl of CCl_4 enriched 45% with $^{13}\text{CCl}_4$ was used. Abbreviation: DPPH, diphenylpicrylhydrazyl.

significant effects on cell viability (measured by lactate dehydrogenase release into the medium and by Trypan Blue exclusion).

The ability of PBN to interfere with the CCl_4 -stimulated lipid peroxidation in isolated hepatocytes is shown in Fig. 7; there is a clear relationship between the intensity of the $\text{PBN-CCl}_3\cdot$ adduct signal and the decrease in lipid peroxidation as measured by the thiobarbituric acid reaction.

Some similar effects of PBN on microsomal activities are given in Table 1, with corresponding data for MNP for comparison. It can be seen that both PBN and MNP inhibit the CCl_4 -stimulated lipid peroxidation when present in concentrations over 2 mM; PBN also strongly inhibits the demethylation of aminopyrene and gives a type I binding spectrum with microsomes from phenobarbital-treated rats. Table 1 also shows that PBN (50 mM) strongly inhibits covalent binding of $^{14}\text{CCl}_4$ to microsomal protein.

Fig. 4. *E.s.r. spectra from experiments in vivo*
Male rats were injected intraperitoneally with 1 ml of a solution containing 17 mg of PBN, and 0.1 ml of CCl_4 in corn oil. After 15 min the animals were killed and the $\text{PBN-CCl}_3\cdot$ adduct was extracted from the liver as described in the Materials and methods section. (a) Control animals injected with PBN alone in corn oil; (c) animals receiving 0.1 ml of CCl_4 in addition to the spin-trap solution mentioned above; (b) animals treated as in (c) but with CCl_4 enriched 45% with $^{13}\text{CCl}_4$. Abbreviation: DPPH, diphenylpicrylhydrazyl.

Table 2 gives results obtained when various free-radical scavengers are included with PBN, CCl_4 and isolated hepatocytes; these compounds are known to inhibit strongly CCl_4 -stimulated lipid peroxidation under these conditions both in isolated hepatocytes and in microsomal suspensions (Poli *et al.*, 1978; Slater & Sawyer, 1971). It can be seen that agents such as promethazine, propyl gallate and (+)-catechin, which all inhibit lipid peroxidation strongly at the concentrations shown in Table 2, had little effect on the PBN adduct formation. The results given in Table 2 for PBN are similar to those reported for MNP by Ingall *et al.* (1978). Inhibitors of cytochrome *P*-450-catalysed metabolism (compound SKF 525A and *p*-chloromercuribenzoate) had no significant effect on the PBN adduct response in microsomes, but gave a small inhibition of e.s.r. signal intensity in hepatocyte suspensions (Table 2). Some small variations in hepatocyte response can be expected, however, as a result of direct action of some of these compounds on the cells. In microsomes the concentrations of compound SKF 525A and *p*-chloromercuribenzoate shown in Table 2 gave stimulation of the covalent binding of $^{14}\text{CCl}_4$ to protein (Cheeseman, 1981). Inhibition of the $\text{PBN-CCl}_3\cdot$ adduct by *p*-chloromercuribenzoate has been obtained in microsomes with higher concentrations of the drug (Poyer *et al.*, 1978), which are, however, unsuitable in isolated liver cells. Table 2 shows that menadione and α -tocopherol at low concentration decreased the PBN adduct signal; the effect is dose-related, as shown in Fig. 8(a). The addition of α -tocopherol to the preformed $\text{PBN-CCl}_3\cdot$ adduct caused a sharp decrease of the e.s.r. signal intensity, probably through a reduction of the adduct. However, the concentrations required are much higher than that

Fig. 5. *Variations in the e.s.r. signal intensity with increasing incubation times and cell concentrations*
For (a) 1.5 ml of cell suspension (10^7 cells/ml) was incubated at 37°C for different times (stated) in the presence of 25 mM-PBN and $10\mu\text{l}$ of CCl_4 placed in the central well of the incubation flasks. For (b) different cell suspensions (concentrations stated) were incubated at 37°C for 10 min with PBN and CCl_4 , as above. All the samples were extracted as described in the text. O, Control suspensions; ●, suspensions incubated in the presence of CCl_4 .

used in the cell suspension. No similar effects have been obtained with menadione (Fig. 8b).

Fig. 6. Variations in the e.s.r. signal intensity of $\text{PBN-CCl}_3\cdot$ adduct with different concentrations of spin trap

Isolated hepatocyte suspensions (10^7 cells/ml) were incubated for 10 min at 37°C with increasing amounts of PBN (stated) and $10\mu\text{l}$ of CCl_4 placed in the central well of the incubation flasks. For experimental details see the text.

Fig. 7. Plot of $\text{PBN-CCl}_3\cdot$ adduct signal intensity versus lipid peroxidation (malondialdehyde production) in isolated hepatocytes treated with CCl_4

Samples (2.5 ml) of hepatocyte suspension (10^7 cells/ml) were incubated for 15 min at 37°C with $10\mu\text{l}$ of CCl_4 in the central well and PBN at the stated concentrations. Portions (1 ml) were extracted for e.s.r. spectra recording ($\blacktriangle$); the remaining part was used for malondialdehyde determination ($\bullet$). For experimental details see the text.

Fig. 8. Decrease in the $\text{PBN-CCl}_3\cdot$ signal intensity induced by α -tocopherol and menadione

(a) Hepatocyte suspensions (10^7 cells/ml) were incubated with CCl_4 and PBN as described for Fig. 3, and different concentrations of α -tocopherol ($\bullet$) and menadione ($\blacksquare$) ($10\mu\text{l}$ of ethanol solutions) were added as shown. (b) Effect of $10\mu\text{l}$ of α -tocopherol ($\bullet$) and menadione ($\blacksquare$) solutions in chloroform added directly to 1 ml of chloroform containing the $\text{PBN-CCl}_3\cdot$ adduct. For experimental details see the text.

Table 1. *Effects of MNP and PBN on several liver microsomal activities*

The systems investigated were: (i) CCl_4 -stimulated lipid peroxidation (Slater & Sawyer, 1971); (ii) $^{14}\text{CCl}_4$ binding to microsomal protein (Cheeseman, 1981); (iii) aminopyrene demethylase activity (Orrenius, 1965); (iv) the difference spectra resulting from the addition of MNP or PBN to one microsomal suspension in a double-beam spectrophotometer experiment (Schenkman *et al.*, 1967). For experimental details see the text. Results are averages of at least two experiments done in triplicate.

System	Concn. of MNP (mM)	Inhibition (%)	Concn. of PBN (mM)	Inhibition (%)
(i)	5	92	5	37
			50	91
(ii)	5	79	5	13
			50	52
(iii)	5	56	5	45
(iv)	Type I		Type I	

Table 2. *Effects of various free-radical scavengers on the e.s.r. intensity of MNP adducts with microsome/ CCl_4 or hepatocyte/ CCl_4 suspensions*

Incubations were for 5–10 min at 37°C under aerobic conditions. For experimental details see the text. The values are given as percentages of the control value in the absence of scavenger. The data for MNP are from Ingall *et al.* (1978).

Scavenger	Effect (% of control value)				
	MNP		PBN		
	Concn. (μM)	Microsomes	Concn. (μM)	Hepatocytes	Microsomes
Promethazine	100	63	100	86	100
Propyl gallate	100	93	100	93	100
(+)-Catechin	100	137	100	100	98
α -Tocopherol	—	—	100	45	57
Menadione	—	—	100	50	20
<i>p</i> -Chloromercuribenzoate	100	92	100	75	96
Metyrapone	—	—	100	96	95
Carbon monoxide	—	41	—	80	75
Compound SKF 525A	100	77	100	74	100

Discussion

The results obtained with MNP show clearly that some product resulting from the metabolism of CCl_4 is trapped; the nitroxyl adduct demonstrated by e.s.r. is dependent on the presence of CCl_4 , NADPH and fresh microsomes. However, the product trapped is not $\text{CCl}_3^{\cdot}$ since no hyperfine splitting could be seen on substituting $^{13}\text{CCl}_4$ for unlabelled CCl_4 . Moreover, the features of the e.s.r. spectrum point clearly to a carbon-centred radical as the trapped species, thereby ruling out $\text{CCl}_3\text{O}_2^{\cdot}$ as a candidate. Results obtained with lipoxxygenase and unsaturated fatty acid point strongly to the conclusion that the species trapped in microsomal suspensions in the presence of NADPH and CCl_4 is a fatty acid radical. The broad lines of the e.s.r. spectrum (Fig. 1b) and the demonstration of more structure in the lipoxy-

genase-formed fatty acid adduct with MNP suggest that the spectrum given in Fig. 1(b) is a composite envelope of a number of similar fatty acid radicals interacting with MNP. Spin trapping of fatty acid radicals has been reported in various studies (De Groot *et al.*, 1973; Kalyanaraman *et al.*, 1979b).

The results found in the photochemically produced $\text{MNP-CCl}_3^{\cdot}$ interaction (see also Symons *et al.*, 1982) show that the $\text{MNP-CCl}_3^{\cdot}$ adduct is unstable and disappears within a few seconds of the incident radiation being stopped. Thus the steady-state concentration of the $\text{MNP-CCl}_3^{\cdot}$ adduct formed in biological systems would be far below the detection limits of the spectrometer.

In contrast with the problems of trapping $\text{CCl}_3^{\cdot}$ with MNP discussed above, the experiments with PBN give unequivocal proof that $\text{CCl}_3^{\cdot}$ is produced in isolated liver cells or in liver microsomes exposed

to CCl_4 . In previous studies we have described the characteristics of the $\text{PBN-CCl}_3^\bullet$ adduct after generating $\text{CCl}_3^\bullet$ by a high-energy radiation procedure under anaerobic conditions (Tomasi *et al.*, 1980; Symons *et al.*, 1982). The adduct formed in isolated hepatocytes with CCl_4 and in microsomes + NADPH + CCl_4 has features under e.s.r. examination identical with those of that formed by direct radiation of CCl_4 in the absence of O_2 (Symons *et al.*, 1982). Moreover, the adduct formed in the biological situations shows clear hyperfine splitting with ^{13}C substitution in CCl_4 , pointing to the presence of the ^{13}C atom β to the nitrogen atom in PBN.

Although radiation studies (Tomasi *et al.*, 1980; Symons *et al.*, 1982) have demonstrated a small but significant change in the $\text{PBN-CCl}_3^\bullet$ adduct when O_2 is present (notably a decrease in the a_N hyperfine splitting constants and lack of ^{13}C hyperfine splitting), no definite evidence could be found for this adduct under biological conditions. The most likely candidate for this particular adduct is the $\text{PBN-CCl}_3^\bullet$ adduct formed via an interaction of the highly electrophilic $\text{CCl}_3\text{O}_2^\bullet$ species (Packer *et al.*, 1978, 1980) and PBN. Possible reasons for not seeing this species trapped in the biological situations where O_2 is certainly present are: (i) that a small proportion of the adduct actually is present but is not capable of detection against a high 'background' of the $\text{PBN-CCl}_3^\bullet$ adduct; (ii) that the much greater reactivity of $\text{CCl}_3\text{O}_2^\bullet$ with amino acids, thiols and polyunsaturated fatty acids would result in unfavourable competition for $\text{CCl}_3\text{O}_2^\bullet$ between such environmental substrates and the relatively low concentration of PBN. Competition kinetics, moreover, indicate a rather slow interaction of $\text{CCl}_3\text{O}_2^\bullet$ with PBN compared with the reactions of $\text{CCl}_3\text{O}_2^\bullet$ with many other species (Tomasi *et al.*, 1980).

A noteworthy feature of the $\text{PBN-CCl}_3^\bullet$ adduct studied in the biological situations reported in the present paper is that the e.s.r. spectra show little difference between the chloroform layer after chloroform/methanol extraction and the results seen in aqueous suspensions of microsomes or hepatocytes without extraction. This result indicates that the $\text{PBN-CCl}_3^\bullet$ adduct remains in a lipid environment even in the aqueous phase trapped in the hydrophobic environment of the membranes.

The results shown in Table 1 indicate some areas of concern that should be carefully evaluated before interpretation of the results obtained with spin traps in biologically complex systems. The spin traps studied in the present work, MNP and PBN, both interact with cytochrome *P*-450 to give difference spectra, and both have inhibitory effects on the mixed-function oxidation of aminopyrene. Thus, in attempting to trap reactive intermediates that are themselves produced by a metabolic activation

involving the NADPH-cytochrome *P*-450 electron-transport chain, it is important not to use concentrations of the spin trap that effectively decrease the metabolic activation necessary for the study. A similar argument can also be presented, for example, in relation to the concentration of CCl_4 used in generating a particular reactive intermediate (in this case $\text{CCl}_3^\bullet$). It is known that the production of $\text{CCl}_3^\bullet$ (and presumably $\text{CCl}_3\text{O}_2^\bullet$, since the reaction of $\text{CCl}_3^\bullet$ with O_2 is essentially diffusion-controlled; Slater, 1982), as judged by the stimulation of lipid peroxidation, increases in relation to the square root of the concentration of CCl_4 over a rather narrow range (Slater & Sawyer, 1971); with high concentrations of CCl_4 the metabolic activity of the NADPH-cytochrome *P*-450 system is strongly depressed.

The effects of free-radical scavengers on the formation of the MNP and PBN adducts (Table 2) are difficult to interpret at first sight, since agents such as promethazine can almost totally block lipid peroxidation, probably because of the initiation event:

In consequence, promethazine might be expected to inhibit strongly the formation of an MNP/PUFA $^\bullet$ adduct, and yet, on the contrary, it has little effect (Table 2; Ingall *et al.*, 1978). Propyl gallate and (+)-catechin behave similarly to promethazine in blocking lipid peroxidation, and in reacting with

Fig. 9. Changes with time in the e.s.r. signal intensity of the MNP adducts formed in liver microsomes incubated with CCl_4 and either placed in a flat cell in the spectrometer cavity (●) or extracted in chloroform/methanol (O)

For experimental details see the text.

Scheme 1. Interactions of the $\text{CCl}_3\cdot$ free radical with O_2 , polyunsaturated fatty acid (PUFA), PBN and promethazine

$\text{CCl}_3\text{O}_2\cdot$ (Slater, 1982), and yet they also have little effect on the MNP-PUFA $\cdot$ adduct. The corresponding results with PBN are less difficult to understand, as PBN essentially traps $\text{CCl}_3\cdot$ (see above) and this radical species does not react rapidly with promethazine (see Slater, 1982). An explanation of the apparently anomalous results with MNP can be obtained through inspection of Fig. 9, which shows that the formation of the MNP-PUFA $\cdot$ adduct in isolated cells, or in microsomal suspensions and without chloroform/methanol extraction, is very slow over the first 10 min of incubation at 37°C. Only when the suspensions are extracted with chloroform/methanol is any rapid reaction apparent. These findings suggest that in the microsomal suspensions, and in the isolated hepatocytes, there is some barrier (either energetic or topographical) that results in a very slow reaction of MNP with PUFA $\cdot$. If this reaction is much slower than the initiation of peroxidation by $\text{CCl}_3\cdot$ (rather than the known fast reaction of $\text{CCl}_3\text{O}_2\cdot$ with PUFA), then the effects of promethazine, propyl gallate and (+)-catechin can be understood. In such a situation lipid peroxidation would be initiated by $\text{CCl}_3\cdot$, which is itself not readily scavenged by promethazine etc. These points are illustrated in Scheme 1.

In relation to this Scheme it is worth noting that promethazine, propyl gallate and (+)-catechin have little influence on covalent binding of $^{14}\text{CCl}_4$ to microsomal protein, presumably operating via the

$\text{CCl}_3\cdot$ radical (Cheeseman, 1981), whereas α -tocopherol strongly inhibits covalent binding and PBN adduct formation. Menadione, which also strongly inhibits PBN adduct formation, has a significant effect on covalent binding of $^{14}\text{CCl}_4$ (Cheeseman, 1981) related to the electron diversion at the flavoprotein site and may also interact with PUFA $\cdot$ via electron donation.

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