SPIN TRAPPING OF FREE RADICAL PRODUCTS OF CCl_4 ACTIVATION USING PULSE RADIOLYSIS AND HIGH ENERGY RADIATION PROCEDURES

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Received 3 November 1980

1. Introduction

Free radicals have been suggested as activated intermediates in many types of tissue injury [1,2]. The techniques of electron spin spectroscopy (ESR) and pulse radiolysis are being increasingly used to characterise the free radicals involved, and to obtain quantitative data on their chemical reactivity [3,4].

Carbon tetrachloride is an important and much studied hepatoxin, which is metabolised by the NADPH-cytochrome P450 electron-transport chain to an activated intermediate generally considered to be the trichloromethyl radical [5,6]. Attempts [7–9] to detect the $CCl_3^{\cdot}$ free radical in whole liver or liver fractions using direct ESR analysis have not been successful, and attention has consequently been directed to the less direct ESR spin trapping techniques [10–12]. Although this procedure is promising for studies both in vitro and in vivo, there are complications in interpretation of the ESR spectra of the free radical species produced in complex biomembrane environments [12,13]. For this reason, we have studied the interaction of $CCl_3^{\cdot}$ with the spin-trap phenylbutylnitrone in a simplified model system where the $CCl_3^{\cdot}$ radicals are produced by a pulse of high energy electrons. We have previously obtained kinetic data on the reactivity of $CCl_3^{\cdot}$ and its peroxy-derivative $CCl_3O_2^{\cdot}$ [14] in this model system. These data, together with ESR analysis with $^{13}CCl_4$ (as suggested in [1]), allow us to unequivocally identify the spin-trap adducts of $CCl_3^{\cdot}$ and $CCl_3O_2^{\cdot}$; identical spectra are also reported for microsomal systems, intact hepatocytes and under conditions in vivo.

2. Materials and methods

CCl_4 was spectroscopic grade (BDH Ltd, Poole, Dorset); phenylbutylnitrone was obtained from Eastman Kodak Co. (Rochester, NY). $^{13}CCl_4$ was purchased from New England Nuclear (Dreieich, FRG). Promethazine was a gift from May and Baker Ltd (Dagenham, Essex). Soya bean lipoxygenase was purchased from Sigma (UK) Ltd.

Trichloromethyl radicals were generated in pure CCl_4 at −196°C using five 200 ns pulses of 4 MeV electron radiation from the Brunel linear accelerator (details in [4]). Irradiation of CCl_4 was carried out:

(i) Anaerobically after repeated freezing-and-thawing cycles of the solutions kept in an anaerobic cabinet containing platinum catalyst and an atmosphere of N_2/H_2;
(ii) With normal dissolved O_2;
(iii) After dropping liquid CCl_4 into liquid O_2 and mechanically mixing the resulting powder.

The oxygen tension was reduced in the 'biological situations' by bubbling the buffers and substrates exhaustively with oxygen-free nitrogen; microsomes and isolated hepatocyte suspensions were treated by blowing a stream of oxygen-free nitrogen across their surfaces at 0°C.

Ultraviolet irradiation was performed with a 400 W lamp with the incident radiation restricted to 300–360 nm by appropriate filters; the lamp was ~5 cm away from the sample and irradiation was for 15 min at room temperature.

ESR analysis was done using a Varian E-3 spectrometer with a variable temperature cavity; the spectrometer settings were: power, 12 mW; modulation, 1 G; scan range, 100 G; temperature, −70°C for 'biological samples', and room temperature for the irradiated samples.

Adult male albino rats (~300 g body wt) were used to prepare microsome and isolated hepatocyte suspensions. The microsomes were prepared as in [15] and were resuspended in 0.15 M KCl: incuba-

tions with CCl_4 were carried out at 37°C in an NADPH-generating system [15]. The amount of CCl_4 added was 2 μl/ml suspension; CCl_4 at ≳10 μl/ml is known to disrupt the organised enzyme system of the endoplasmic reticulum and this may have affected the results in [12]. Isolated hepatocytes (15×10^6 cells/ml final mixture) were prepared and incubated with CCl_4 as in [16]. PBN was added to the incubation mixtures at 25 mM. After the incubation of microsomes or isolated hepatocytes, an extraction into chloroform:methanol (2:1, v/v) was performed; the chloroform layer was used for ESR.

3. Results and discussion

Irradiation of pure CCl_4 with high energy electrons at −196°C in the absence of O_2 (or presence of O_2) resulted in the appearance of a simple ESR spectrum with only minor hyperfine components. Warming the frozen irradiated samples to ~−70°C with subsequent re-cooling led to much more complex ESR spectra with marked hyperfine components. In the anaerobic case, the re-cooled sample gave spectra identical with the features described for $CCl_3^{\cdot}$ in [17,18]. Substitution with ^{13}C was accompanied by easily observable ^{13}C hyperfine components identical with the ^{13}C spectra in [18,19]. When the irradiation, warming-up and re-cooling were done in the presence of O_2, the ESR spectrum showed differences to the spectrum of the anaerobic samples; in particular, signal broadening and decreased hyperfine complexity that were consistent with the report [18] for the $CCl_3O_2^{\cdot}$ radical. A rather similar spectrum has been reported for the corresponding $CF_3O_2^{\cdot}$ radical [20]. Thus, the irradiation procedure used here produces both types of free radical required for our model experiments: $CCl_3^{\cdot}$ anaerobically, and $CCl_3O_3^{\cdot}$ aerobically on warming to ~−70°C.

With a frozen anaerobic irradiated sample of CCl_4 containing PBN (molar ratio, 1000:1) the characteristic $CCl_3^{\cdot}$ spectrum was observed between −196°C and −70°C; ^{13}C substitution produced the same effects as described above. Warming the anaerobic frozen sample to room temperature produced marked time-related changes in the spectrum that finally stabilised after ~10 min to a triplet of doublets (fig.1A: N-splitting, 14 G; H-splitting, 1.8 G). This triplet of doublets was split by ^{13}C substitution (fig.1B). The intensity of the split lines was consistent with the percentages of ^{13}C present, and shows that the triplet of doublets must represent mainly one radical species, almost certainly the CCl_3–PBN adduct.

When a similar mixture of CCl_4 and PBN was exposed to the electron pulse at −196°C in the pres-

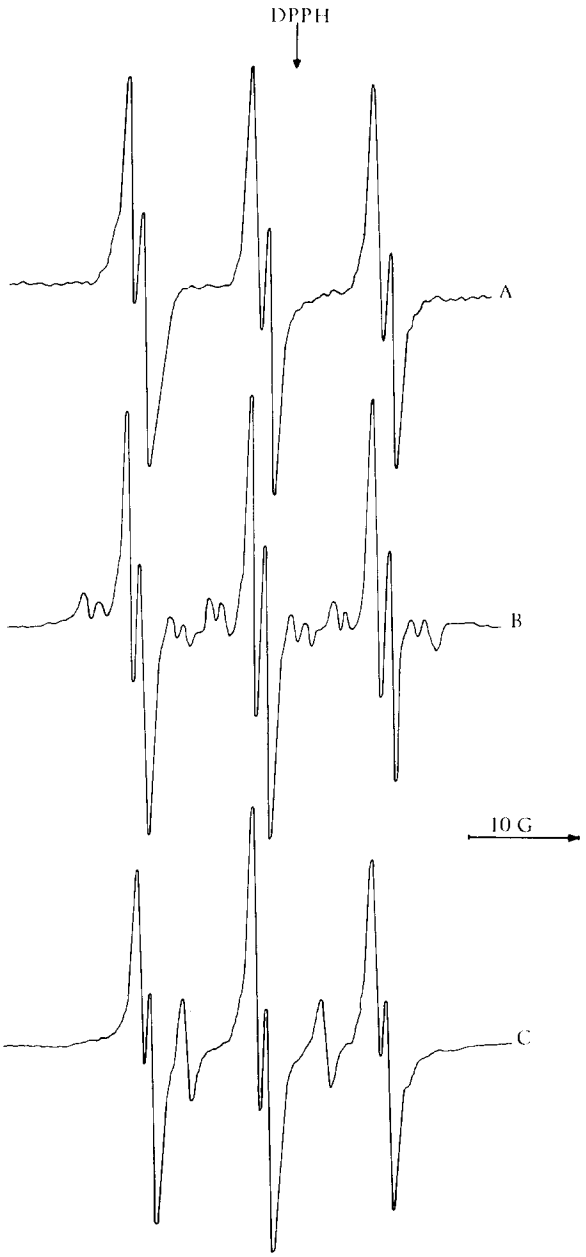

Fig.1. (A) CCl_4 + PBN (25 mM) irradiated by 5 pulses of a high energy electron beam (10 krad) under strictly anaerobic conditions. (B) Same conditions as (A) but including $^{13}CCl_4$ at 10%. (C) Same conditions as (A) but in presence of oxygen.

ence of O_2, the ESR spectrum was identical with that of $CCl_3^{\cdot}$ in the range −196°C and −70°C. On warming, there was again a transient disturbance in the spectrum that finally stablised (over 10 min) to a triplet (N-splitting, 7.9 G) and a triplet of doublets (fig.1; N-splitting, 13 G; H-splitting, 1.63 G). The narrow triplet is most probably the benzoyl–PBN product caused by oxidative side reactions [21]. The triplet of doublets we ascribe to the $CCl_3O_2^{\cdot}$–PBN adduct; no ^{13}C-splitting was seen as the ^{13}C would be δ to the nitroxy group. It is noteworthy that the hyperfine splitting in the presence of O_2 is narrower than in its absence; this is consistent with the results in [22] for alkyl peroxy radicals. The unstable species that appeared during the warming up from −60°C to room temperature appears to be a chlorine atom adduct of PBN (N-splitting, 11.9 G; H-splitting, 0.75 G; ^{37}Cl-splitting, 4.9 G; ^{35}Cl-splitting, 5.9 G).

The same final ESR spectra as in fig.1 were produced on irradiating CCl_4–PBN mixtures at 300–360 nm at room temperature. The hyperfine splittings were identical to those reported above, and ^{13}C-splitting was observed only in the absence of O_2.

The rate of reaction of PBN with $CCl_3^{\cdot}$ and $CCl_3O_2^{\cdot}$ was examined by competition methods and pulse radiolysis [4]. Using the $CCl_3O_2^{\cdot}$–promethazine reaction as the basis for competition [14] it was found that PBN reacts with $CCl_3O_2^{\cdot}$ with a rate constant of $\sim 5.4 \times 10^6\ M^{-1} \cdot s^{-1}$. The reaction of $CCl_3^{\cdot}$ with PBN was too slow to measure by the pulse radiolysis procedure and its rate constant is probably $<10^4\ M^{-1} \cdot s^{-1}$.

When microsomes or isolated hepatocytes were incubated with CCl_4 and PBN aerobically (and also anaerobically for microsomes) the same spectrum as in fig.1A was obtained (fig.2B); strong ^{13}C-splitting was observable (fig.2C). With increased O_2 tension (produced by bubbling pure O_2 into the tissue suspensions) the ESR signal heights decreased and the triplet of doublets found in the presence of CCl_4 and PBN had different hyperfine splitting constants (N-splitting, 13.6 G; H-splitting, 2.2 G). There were no effects produced by ^{13}C substitution in the presence of high O_2 levels and the spectrum was identical to that produced on interacting PBN with peroxidising arachidonic acid, and on mixing PBN with arachidonic acid and soya bean lipoxygenase (fig.2D).

The $CCl_3^{\cdot}$ and $CCl_3O_2^{\cdot}$ species formed by electron radiation of frozen CCl_4 clearly react with PBN at −50–0°C to yield rather stable characteristic adducts as shown in fig.1A,C. Interference from secondary radicals (e.g., from PBN) is low due to the high concentration of CCl_4 relative to PBN in our system. The spectrum of the adduct from $CCl_3^{\cdot}$ and PBN, formed in the absence of O_2 splits as expected when $^{13}CCl_4$ is used and this allows us to define this product as the CCl_3–PBN adduct. The nitrogen and hydrogen hyperfine values are identical with those in [23]

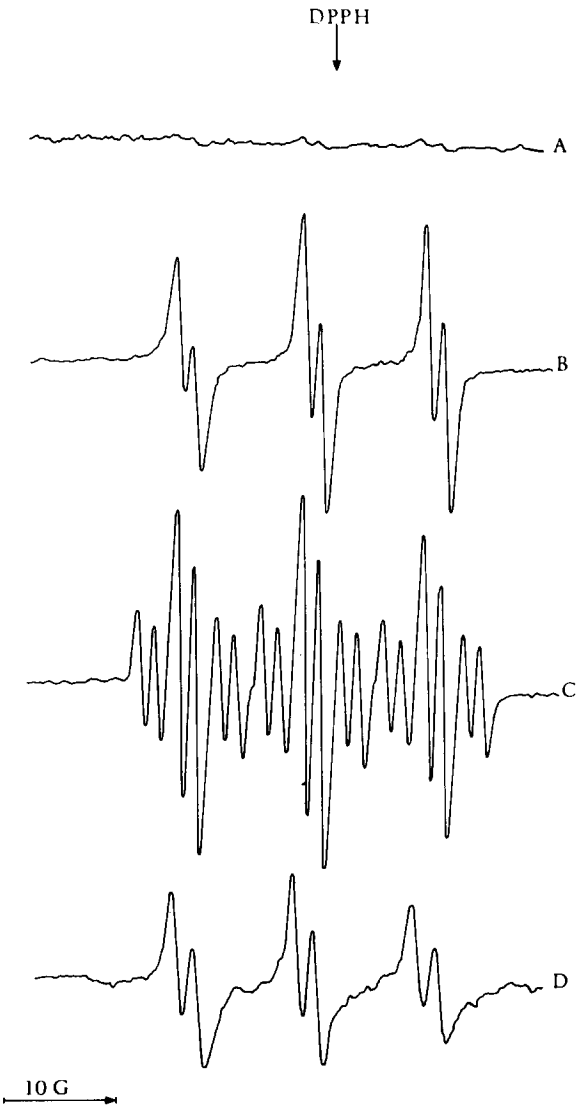

Fig.2. (A) Hepatocytes suspension incubated with PBN (25 mM) and NADPH (1 mM) for 10 min at 37°C. (B) Same conditions as (A) but 10 μl CCl_4 in the central well. (C) Same conditions as (B) but $^{13}CCl_4$ at 25%. (D) Same conditions as (B,C) but oxygen bubbled in the cell suspension for 10 min before the incubation with CCl_4.

within experimental accuracy. Our model, however, allows us additionally to characterise the spectrum of the $CCl_3O_2^{\cdot}$–PBN adduct and, as mentioned above, the hyperfine values are significantly changed compared to the $CCl_3^{\cdot}$–PBN adduct.

Since the spectrum of the CCl_3O_2–PBN adduct was not detected in the aerobically incubated suspensions of microsomes or hepatocytes it might be considered that the $CCl_3O_2^{\cdot}$ species is not formed in such biological environments despite the very high rate constant for the reaction of $CCl_3^{\cdot}$ with O_2 [14,24]. A partial explanation for the failure to detect the CCl_3O_2–PBN adduct may be that the concentration of O_2 is rapidly depleted in the endoplasmic reticulum under conditions in vitro; however, increasing the oxygen partial pressure, as mentioned earlier, did not result in a spectrum of the CCl_3O_2–PBN adduct but to a lipid-type radical–PBN adduct probably derived from lipid peroxidation. This latter finding suggests that the failure to observe the CCl_3O_2–PBN adduct in the biological systems is due to the high chemical reactivity of $CCl_3O_2^{\cdot}$ with polyunsaturated fatty acids, amino acids and thiol groups [25]: thus, the local concentration of PBN may be far too small for effective competition to trap the $CCl_3O_2^{\cdot}$ species.

These data, which supplement independent studies [11,23], clearly demonstrate that the $CCl_3^{\cdot}$ free radical is produced in biological systems and can be trapped with PBN. In addition, the ESR spectrum of the $CCl_3O_2^{\cdot}$–PBN adduct shows that a more reactive derivative of $CCl_3^{\cdot}$ can also be trapped, at least in model systems. In biological environments the $CCl_3O_2^{\cdot}$ species appears to be too reactive for trapping by PBN and, in consequence, only secondary lipid radicals are trapped. These data have obvious bearing on the biological consequences of CCl_4 activation, and on possible mechanisms of protection.

Acknowledgements

We are grateful for financial support from the Wellcome Trust, London; the Fondazione Bossolasco, Torino; and the National Foundation for Cancer Research. We are also most grateful to Professor M. C. R. Symons for helpful advice on interpretation of the ESR spectra, and expert evaluation of the PBN adducts.

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