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# Free-radical metabolism of carbon tetrachloride in rat liver mitochondria

## A study of the mechanism of activation

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Alterations in liver mitochondria as consequence of rat poisoning with carbon tetrachloride ($CCl_4$) have been reported over many years, but the mechanisms responsible for causing such damage are still largely unknown. Isolated rat liver mitochondria incubated under hypoxic conditions with succinate and ADP were found able to activate $CCl_4$ to a free-radical species identified as trichloromethyl free radical ($CCl_3^{\cdot}$) by e.s.r. spectroscopy coupled with the spin-trapping technique. The incubation of mitochondria in air decreased free-radical production, indicating that a reductive reaction was involved in the activation of $CCl_4$. However, in contrast with liver microsomes (microsomal fractions), mitochondria did not require the presence of NADPH, and the process was not significantly influenced by inhibitors of cytochrome *P*-450. The addition of inhibitors of the respiratory chain such as antimycin A and KCN decreased free-radical formation by only 30%, whereas rotenone displayed a greater effect (approx. 84% inhibition), but only when preincubated for 15 min with mitochondria not supplemented with succinate. These findings suggest that the mitochondrial electron-transport chain is responsible for the activation of $CCl_4$. A conjugated-diene band was observed in the lipids extracted from mitochondria incubated with $CCl_4$ under anaerobic conditions, indicating that stimulation of lipid peroxidation was occurring as a result of the formation of free-radical species.

## INTRODUCTION

Carbon tetrachloride ($CCl_4$) is probably the most studied hepatotoxin, and its activation by microsomal enzymes has been known for a long time (Slater, 1972). Only recently, however, has the formation of trichloromethyl free radical ($CCl_3^{\cdot}$) been directly demonstrated by the use of e.s.r. spectroscopy coupled with the spin-trapping technique (Poyer *et al.*, 1980; Tomasi *et al.*, 1980).

Early reports concerning the pathological effects of $CCl_4$ showed that the mitochondria were altered after $CCl_4$ poisoning of rats (Christie & Judah, 1954; Dianzani, 1954). Uncoupling of oxidative phosphorylation and impairment of $Ca^{2+}$ transport were described as consequence of the exposure to haloalkane (Dianzani, 1954; Carafoli & Tiozzo, 1968; Slater & Delaney, 1970).

Experiments *in vitro* with isolated hepatocytes have shown that alterations in mitochondrial $Ca^{2+}$ uptake are an early effect on $CCl_4$ poisoning and do not depend upon the stimulation of lipid peroxidation induced by $CCl_4$ within the cells (Albano *et al.*, 1985).

The possibility that $CCl_4$ might be activated in the mitochondria has been suggested by the recovery of alkylated proteins and lipids extracted from highly purified mitochondria incubated with $CCl_4$ (De Castro *et al.*, 1984). Moreover, covalently bound $CCl_4$ has been detected in mitochondrial DNA after poisoning *in vivo* of rats, and the ratio of radiolabelled nucleotides in mDNA was 20–50-fold higher than in the nuclear DNA obtained from the same livers (Levy & Brabec, 1984). The existence of such an activation process could explain the above-mentioned alterations in mitochondrial function.

An unambigous demonstration of $CCl_4$ metabolism in mitochondria was attempted by the spin-trapping technique, previously used for the detection of $CCl_3^{\cdot}$ in isolated hepatocytes and liver microsomes (Tomasi *et al.*, 1980; Albano *et al.*, 1982).

## MATERIAL AND METHODS

PBN was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; [$^{13}$C]$CCl_4$ was from New England Nuclear Corp. (Dreieich, Germany); SKF 525A was kindly provided by Smith, Kline and French (Welwyn Garden City, Herts., U.K.); *p*-CMB, metyrapone, ADP, antimycin A, rotenone and FCCP were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Boehringer (Mannheim, Germany).

Adult male Wistar rats (Nossan, Correzana, Italy) weighing approx. 200 g were maintained on a standard laboratory diet (Piccioni, Brescia, Italy) and water *ad libitum*; animals were fasted for 12 h before they were killed.

Rat liver mitochondria were isolated by differential centrifugation using a standard procedure (Muscatello *et*

Abbreviations used: $CCl_3^{\cdot}$, trichloromethyl free radical; PBN, phenyl-t-butyl nitrone; SKF 525A, 2-diethylaminoethyl 3,3-diphenylpropylacetate; *p*-CMB, *p*-chloromercuribenzoate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

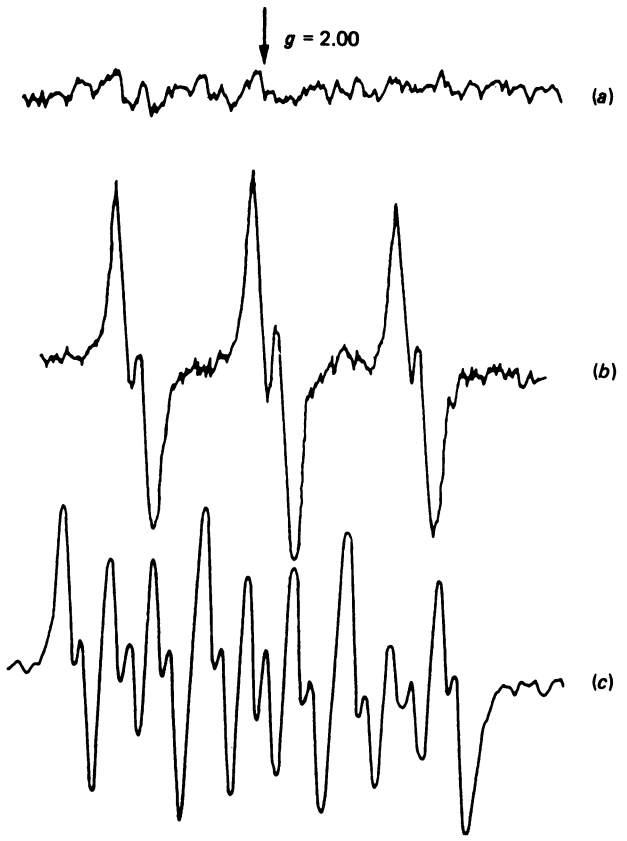



**Fig. 1. E.s.r. spectra of the $CCl_3^{\cdot}$–PBN free-radical adduct produced by isolated mitochondria exposed to $CCl_4$**

Mitochondrial suspensions were incubated 30 min at 37 °C under hypoxic conditions with 0.5 mM-ADP, 5 mM-succinate, 25 mM-PBN and in the presence of $CCl_4$ (10 $\mu$l added to the centre well of the incubation flasks). The traces represent: (*a*) controls incubated without $CCl_4$; (*b*) mitochondria receiving $CCl_4$; (*c*) mitochondria incubated with $CCl_4$ enriched 60% with the $^{13}C$ radioisotope.

*al.*, 1972). Protein concentration was determined by the biuret reaction. Mitochondria (10 mg of protein/ml) were resuspended in a medium containing 100 mM-NaCl, 10 mM-$MgCl_2$, 10 mM-Tris/HCl buffer, pH 7, 10 mM-sodium/potassium phosphate buffer, pH 7.4, and 25 mM-PBN as spin trap. ADP (0.5 mM) and succinic acid (5 mM) were added, when indicated, at the beginning of the incubation.

Spin-trapping experiments were performed on freshly prepared and resuspended mitochondria (2 ml final volume) placed in a 50 ml Erlenmeyer flask fitted with a central well. $CCl_4$ (10 $\mu$l) was added to the central well and the flasks were tightly closed with silicone septa. This procedure allows the diffusion of sufficient amounts of $CCl_4$ (approx. 0.15 mM) (Poli *et al.*, 1979) into the incubation mixture, avoiding the damage of mitochondria caused by the solvent properties of $CCl_4$. Inhibitors were dissolved in water or dimethylformamide and small volumes (50 or 5 $\mu$l respectively) were added to the incubation mixture.

Hypoxic conditions were obtained by flushing the mitochondrial suspension for 10 min with moist $O_2$-free $N_2$. Incubations were carried out at 37 °C for 30 min, after which the suspensions were extracted with 1 ml of

**Table 1. Effect of various treatments on the formation of $CCl_3$–PBN free-radical adduct in isolated mitochondria**

Liver mitochondria were incubated for 30 min at 37 °C under hypoxic conditions and, if not otherwise stated, they were energized with succinate and ADP as reported in the Materials and methods section. The values are expressed in arbitrary units and represent the means (±S.E.M.) for at least four different experiments. Significance was estimated by Student's *t* test: [a]$P > 0.01$; [b]$P > 0.5$; [c]not significant.

| Treatment | E.s.r. signal intensity | As % of control |
|---|---|---|
| None (control) | 7.87±0.78 | 100 |
| +Antimycin A (1 $\mu$g/ml) | 5.69±1.60[b] | 71 |
| +KCN (1 mM) | 5.70±1.10[b] | 72 |
| +FCCP (1 $\mu$M) | 9.86±2.17[c] | 120 |
| +Rotenone (6 $\mu$M) | 7.80±1.12[c] | 99 |
| −ADP and succinate | 1.25±0.72[a] | 16 |
| −ADP and succinate | 7.80±0.84[c] | 99 |
| Incubation in air | 4.46±0.84[a] | 57 |
| Heat-inactivated mitochondria | 0 | 0 |

chloroform/methanol (2:1, v/v) mixture, and the chloroform phase separated by centrifugation was used for e.s.r. analysis.

The presence of nitroxide spin adducts was revealed by a Bruker 200D-SCR e.s.r. spectrometer as previously described (Albano *et al.*, 1982).

Diene conjugation absorbances were determined on the lipids extracted from mitochondria incubated with $CCl_4$ as described above, but without PBN, as previously described (Corongiu *et al.*, 1983). Briefly, lipids were extracted with 5 ml of chloroform/methanol mixture, the chloroform phase dried under vacuum and resuspended in cyclohexane to a concentration of 25 $\mu$g/ml. Samples were scanned from 300 to 200 nm with a Perkin–Elmer 500-S double-beam spectrometer set up in the second-derivative mode (Corongiu *et al.*, 1983).

## RESULTS

Incubation, under hypoxic conditions, of liver mitochondria in a medium containing succinic acid, ADP, PBN and in the presence of $CCl_4$ resulted in the formation of a PBN spin adduct showing the following spectral features: $a_N = 1.40$ mT (14.0 G); $a_H = 0.175$ mT (1.75 G) (Fig. 1*b*). The introduction of $CCl_4$ (carbon tetrachloride) labelled with $^{13}C$ produced a further splitting in the e.s.r. spectrum [$a^{13}C = 0.97$ mT (9.6 G)] (Fig. 1*c*), which confirmed that the radical trapped originated from the $CCl_4$ molecule and allowed its identification as $CCl_3^{\cdot}$ (Albano *et al.*, 1982).

No e.s.r. signals were observed when $CCl_4$ was omitted from the incubation mixture (Fig. 1*a*). Similarly, boiling the mitochondrial suspension for 15 min before incubation completely abolished the formation of $CCl_3^{\cdot}$ (Table 1).

After the incubation of mitochondria with $CCl_4$ in air, the formation of the PBN adduct was still detectable, although it was decreased by approx. 50% (Table 1).

To exclude the possibility that the formation of $CCl_3^{\cdot}$

**Table 2. Effect of several inhibitors of microsomal mono-oxygenase activity on the free-radical activation of $CCl_4$ by liver mitochondria**

Liver mitochondria were incubated under hypoxic conditions as described in the Materials and methods section. The values are expressed in arbitrary units and represent the means (±S.E.M.) for five different experiments. Significance was estimated by Student's $t$ test: [a]$P > 0.01$; [b]$P > 0.05$; [c]not significant.

| Treatments | E.s.r. signal intensity | As % of control |
|---|---|---|
| None (control) | 7.87±0.78 | 100 |
| +SKF 525A (1 mM) | 7.41±0.65[c] | 94 |
| +Metyrapone (0.2 mM) | 6.40±1.70[c] | 81 |
| +$p$-CMB (0.1 mM) | 7.15±0.70[c] | 91 |
| +$TlCl_3$ (0.2 mM) | 4.30±0.95[a] | 56 |

be attributable to the contamination of our mitochondria preparations by microsomal membranes or, alternatively, by the small amount of cytochrome $P$-450 present in these organelles (Niranjani *et al.*, 1984), experiments were carried out with inhibitors of the microsomal mono-oxygenase system. As reported in Table 2, the addition of either 1 mM-SKF 525A, 0.5 mM-metyrapone or 0.1 mM-$p$-CMB did not significantly affect the production of $CCl_3^{\cdot}$.

The activation of $CCl_4$ is known to require, as an initial event, the transfer of one electron to form the $CCl_4^-$ anion, and any source of electrons having suitable potential could theoretically catalyse the process (Slater, 1972; McDonald, 1982). It is therefore possible that the mitochondrial respiratory chain can supply the electrons required for the reduction of $CCl_4$.

The incubation of mitochondria under hypoxic conditions in the presence of antimycin A (1 g/ml) or KCN (1 mM) decreased by approx. 30% the intensity of the signal due to the $CCl_3^{\cdot}$–PBN adduct (Table 1). The addition of rotenone (6 $\mu$M) strongly inhibited the activation of $CCl_4$, but only when it was preincubated for 15 min with mitochondria not supplemented with succinic acid and ADP (Table 1). The simple omission of succinic acid and ADP, however, did not in itself interfere with free-radical formation (Table 1), probably because endogenous substrates were sufficient for carrying out the reaction during 30 min incubation.

The uncoupling of oxidative phosphorylations by FCCP had no significant effect on the formation of $CCl_3^{\cdot}$–PBN spin adducts (Table 1).

The mitochondrial activation of $CCl_4$ was instead decreased by thallium chloride ($TlCl_3$) (0.2 mM), a thiol-reactive reagent that also inhibits microsomal flavoproteins (Woods & Flower, 1985).

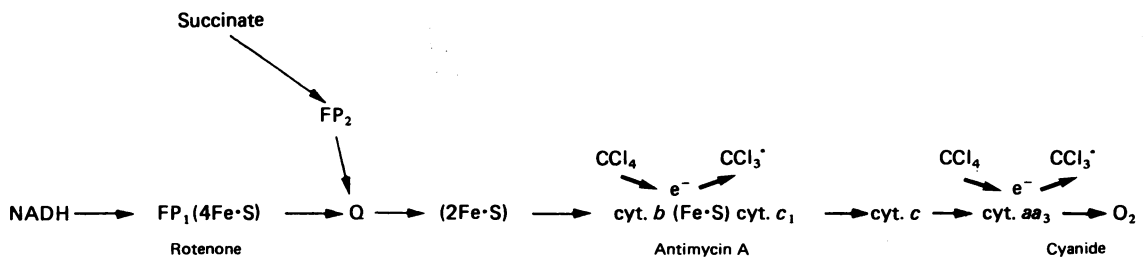
The stimulation of lipid peroxidation resulting from the formation of $CCl_3^{\cdot}$ has been evaluated by measuring the conjugated-diene absorbance in mitochondrial lipids. As shown in Scheme 1, conjugated dienes were observed only in the lipids extracted from mitochondria incubated with $CCl_4$ under hypoxic conditions, in accordance with the increased free-radical production observed at low $O_2$ partial pressures. Conversely, no diene absorbance was detected in mitochondria incubated in the absence of $CCl_4$ or when the haloalkane was added to normoxic mitochondrial preparations (Figs. 2*a* and 2*b*).

## DISCUSSION

Liver injury induced by $CCl_4$ is characterized by the impairment of several cellular functions, including those of mitochondria. The mechanisms responsible for causing damage of mitochondria are still largely unknown, but it is unlikely that $CCl_3^{\cdot}$ produced by microsomal cytochrome $P$-450 can diffuse so far away from its site of formation to interact with these organelles (Slater, 1976). On the other hand, the spreading within the cells of products originating from lipid peroxidation does not seem to be responsible for the mitochondrial damage (Albano *et al.*, 1985).

The present study shows that mitochondria themselves are able to activate $CCl_4$ to a free-radical species having spectral features identical with those of the $CCl_3^{\cdot}$–PBN adduct detected in isolated hepatocytes or liver microsomes (Poyer *et al.*, 1980; Tomasi *et al.*, 1980; Albano *et al.*, 1982). The observation of the hyperfine splitting due to the $^{13}C$ radioisotope confirms that $CCl_3^{\cdot}$ is the free-radical species trapped by PBN (Tomasi *et al.*, 1980).

In the mitochondria, as in the liver microsomes, low $O_2$ partial pressures stimulate free-radical formation, suggesting that a one-electron reductive process is taking place (Slater, 1972). In contrast with microsomes, however, mitochondria generate the $CCl_3^{\cdot}$–PBN adducts in the absence of added NADPH, and such a process is not influenced by several inhibitors of microsomal mono-oxygenase systems which are, instead, effective in decreasing the free-radical activation of $CCl_4$ by microsomes (Cheeseman *et al.*, 1985). In contrast, the formation of $CCl_3^{\cdot}$ is affected by inhibitors of the mitochondrial electron-transport chains, such as rotenone, antimycin and KCN, as well as by the thiol-reactive




**Scheme 1. Scheme representing the mitochondrial electron-transport chain and showing the proposed activation sites of $CCl_4$**

Abbreviations used: FP, flavoprotein; Fe·S, iron–sulphur protein; Q, ubiquinone; cyt., cytochrome.

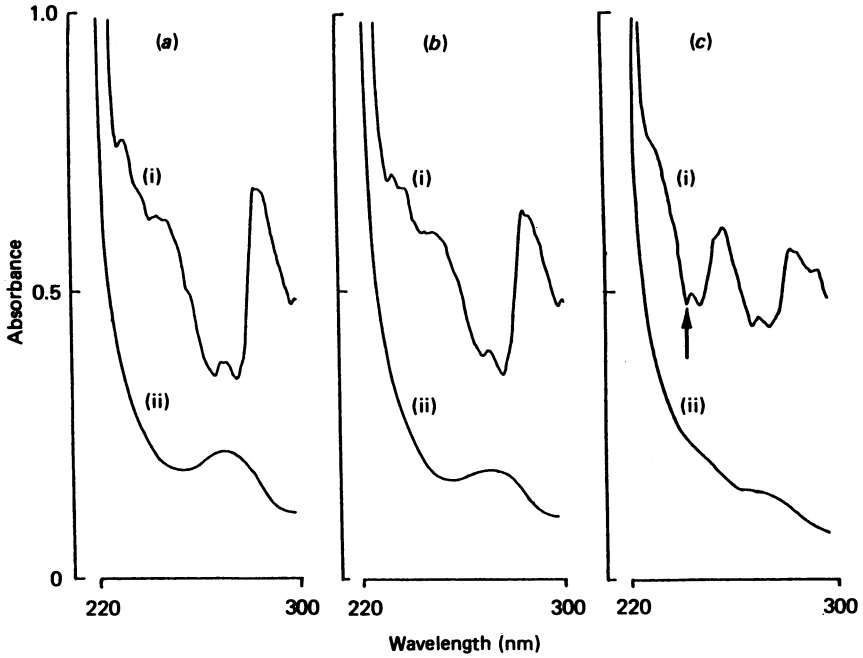



**Fig. 2. Absorption due to conjugated-diene bands in the lipids extracted from isolated liver mitochondria incubated with or without $CCl_4$**

Mitochondrial suspensions were incubated under the same conditions reported for spin-trapping experiments, except that PBN was omitted. At the end of the incubation, lipids were extracted as described by Corongiu *et al.* (1983) and resuspended in cyclohexane at a concentration of 25 $\mu$g/ml. The spectra were recorded as simple absorption (trace ii) or the second derivative (trace i). (*a*) Refers to control mitochondria incubated without $CCl_4$; (*b*) and (*c*) show the spectra obtained from mitochondria exposed to $CCl_4$ under normoxic and hypoxic conditions respectively.

reagent thallium chloride, which probably interferes with iron–sulphur complexes of the respiratory chain.

With respect to the mechanisms responsible for the activation of $CCl_4$, we suggest that it might share some analogies with the univalent reduction of oxygen occurring along the respiratory chain (Forman & Boveris, 1982).

$CCl_4$, in fact, has a reduction potential close to that of $O_2$ (Hanzlik, 1982) and therefore it could compete with $O_2$ for the reducing equivalents.

The data so far available suggest that probably both Complex III and IV of the mitochondrial electron-transport chain participate to the activation of $CCl_4$. In favour of a role of Complex III are the partial inhibition of the e.s.r. signal by antimycin A and the similarity of the electrochemical potential of the complex (Hatefi, 1985) with the reduction potential of $CCl_4$ (Fry, 1972). However, a similar degree of inhibition is also produced by KCN. This result is suggestive of cytochrome $a_3$ being involved in the reduction process, probably by a mechanism not dissimilar from that of cytochrome *P*-450-mediated activation.

The production of $CCl_3^{\bullet}$ radicals by the respiratory chain could explain why $^{14}CCl_4$ residues were found covalently bound to mDNA (Levy & Brabec, 1984), which is located in the internal matrix of mitochondria (Goodenough & Levine, 1970) and can be reached only by reactive intermediates produced in its close proximity.

The free-radical activation of $CCl_4$ within the mitochondria is associated with the detection of conjugated dienes, indicating the occurence of peroxidative alterations in the organelle membranes. The stimulation of lipid peroxidation along with macromolecule alkylation can be postulated as possible causes for the impariment of mitochondrial functions induced by $CCl_4$.

The demonstration of such a peculiar mechanism in $CCl_4$ activation by mitochondria could lead one to reconsider the role played by the damage of these organelles in the pathogenesis of $CCl_4$ toxicity.

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