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Carbon Tetrachloride Toxicity as a Model for Studying Free-Radical Mediated Liver Injury [and Discussion]

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Carbon tetrachloride toxicity as a model for studying free-radical mediated liver injury

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A single dose of CCl_4 when administered to a rat produces centrilobular necrosis and fatty degeneration of the liver. These hepatotoxic effects of CCl_4 are dependent upon its metabolic activation in the liver endoplasmic reticulum to reactive intermediates, including the trichloromethyl free radical. Positive identification of the formation of this free radical *in vivo*, in isolated liver cells and in microsomal suspensions *in vitro* has been achieved by e.s.r. spin-trapping techniques. The trichloromethyl radical has been found to be relatively unreactive in comparison with the secondarily derived peroxy radical $CCl_3O_2^{\cdot}$, although each free radical species contributes significantly to the biological disturbances that occur. Major early perturbations produced to liver endoplasmic reticulum by exposure *in vivo* or *in vitro* to CCl_4 include covalent binding and lipid peroxidation; studies of these processes occurring during CCl_4 intoxication have uncovered a number of concepts of general relevance to free-radical mediated tissue injury. Lipid peroxidation produces a variety of substances that have high biological activities, including effects on cell division; many liver tumours have a much reduced rate of lipid peroxidation compared with normal liver. A discussion of this rather general feature of liver tumours is given in relation to the liver cell division that follows partial hepatectomy.

Introduction

It has been known for more than 100 years that carbon tetrachloride is a very toxic substance. However, although its damaging actions on the liver were recorded in the early literature, the detailed analysis of its hepatotoxic actions can be traced back mainly to the series of studies by Cameron and his colleagues, commencing in the 1930s (Cameron & Karunaratne 1936). When powerful new techniques of biochemistry became available in the period 1950–1960 there was a proliferation of biochemical and toxicological studies on CCl_4 because it was widely recognized to have features making it well suited as a model agent for studying hepatotoxicity. These features include its ready availability in pure form, the reproducibility of its effects on liver in different species, and the diversity of effects it can produce under appropriate conditions. Most studies have concentrated on the early acute effects of CCl_4 on rat liver: a single dose of CCl_4 (for example, 0.5 ml per kilogram of body mass) administered to a rat causes centrilobular necrosis and fatty degeneration of the liver. Repeated doses of CCl_4 can lead to the onset of cirrhosis and, under certain conditions, to liver tumours. In this paper we will concentrate mainly on the early acute effects of CCl_4 on the liver, but some remarks germane to liver tumours will be included at the end.

Butler (1961) and an associated commentary by Wirtschafter & Cronyn (1964) suggested that CCl_4 was converted to a trichloromethyl radical ($CCl_3^{\cdot}$) that was of significance to the ensuing lesion. In the data and discussions of those early papers there were no indications of

any specific metabolic route for the production of $CCl_3^{\bullet}$ from CCl_4. The suggestion that CCl_4 was converted to $CCl_3^{\bullet}$ in liver was developed by Slater (1966) into the concept of the metabolic activation of CCl_4 by enzymes in liver endoplasmic reticulum with toxic consequences such as lipid peroxidation. Almost simultaneously, and quite independently, Recknagel and coworkers (Ghoshal & Recknagel 1965) considered the formation of $CCl_3^{\bullet}$ from CCl_4, and also stressed the importance of lipid peroxidation, initiated and stimulated by $CCl_3^{\bullet}$, in the liver damage. Since the publications of Slater (1966) and of Ghoshal & Recknagel (1965) many other studies have confirmed the metabolic activation of CCl_4, its relevance to liver injury, and its association with the NADPH–cytochrome-P_{450} electron-transport chain. There is no intention here to review basic features of CCl_4-mediated liver injury; full reviews of the general background can be obtained in Recknagel (1967) and Slater (1972).

In this paper we will consider some key aspects of the liver injury caused by CCl_4 that are still in doubt or controversial; we will outline a number of concepts of general importance in relation to biochemical studies on tissue injury, and which have been derived largely from work with CCl_4; the toxicological significance of lipid peroxidation with respect to CCl_4-induced liver injury will be briefly discussed; and some of our recent studies on lipid peroxidation in relation to cell division and cancer will also be described.

Metabolic activation and free-radical products

Although many early biochemical studies were based upon the working hypothesis that CCl_4 was metabolically activated to $CCl_3^{\bullet}$ in the endoplasmic reticulum of liver, it took a relatively long time to obtain unequivocal evidence that this was indeed so. Experiments (Ingall *et al.* 1978) to demonstrate the formation of $CCl_3^{\bullet}$ in whole liver or in liver microsomes by direct electron spin resonance (e.s.r.) spectroscopy were not successful, probably because the concentration of $CCl_3^{\bullet}$ was too low for the sensitivity of the method, and because of the well known fact that liver samples examined by e.s.r. show an envelope of overlapping signals close to $g = 2$, where the absorption of $CCl_3^{\bullet}$ would occur. Attempts were then made to establish the occurrence of $CCl_3^{\bullet}$ by the less direct method of e.s.r. spin trapping, but no clear evidence was obtained by using *N*-methyl-nitrosopropane (Ingall *et al.* 1978). McCay's group introduced the spin trap phenylbutyl nitrone (PBN) for biological use and obtained good evidence for the production of $CCl_3^{\bullet}$ from CCl_4 in liver microsomes and in liver *in vivo* (Poyer *et al.* 1978, 1980). Consistent and complementary studies (Albano *et al.* 1982) have provided unequivocal evidence for the formation of $CCl_3^{\bullet}$ by liver microsomes plus NADPH, in isolated hepatocytes and *in vivo*. These spin-trapping studies, together with radioisotope labelling analysis of covalently bound products, and the formation of hexachloroethane and chloroform (for background see, for example, Slater 1972) establish beyond doubt that $CCl_3^{\bullet}$ is a 'normal' metabolite of CCl_4 in rat liver.

It is known that $CCl_3^{\bullet}$ reacts quickly with oxygen to yield the trichloromethyl peroxy radical $CCl_3O_2^{\bullet}$ (Packer *et al.* 1978) and that the $CCl_3O_2^{\bullet}$ radical is much more reactive chemically than $CCl_3^{\bullet}$. Attempts to trap $CCl_3O_2^{\bullet}$ in liver systems are hindered by the lability of peroxy adducts (Niki *et al.* 1983) and the high reactivity of $CCl_3O_2^{\bullet}$ with molecules in its immediate environment. Direct evidence for the interaction of $CCl_3^{\bullet}$ and O_2 has been obtained by low-temperature e.s.r. experiments in chemical model systems (see, for example, Symons *et al.* 1982), but a direct demonstration in liver samples has not yet been achieved. In recent work

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a secondarily derived peroxy species has been trapped (M. Davies, K. A. K. Lott & T. F. Slater 1985, unpublished results), but this is probably the lipid peroxy radical resulting from the interaction of $CCl_3O_2^{\cdot}$ with a polyunsaturated fatty acid PUFA:

$$CCl_3O_2^{\cdot} + PUFA \rightarrow PUFA^{\cdot} + CCl_3O_2H,$$

$$PUFA^{\cdot} + O_2 \rightarrow PUFAO_2^{\cdot}.$$

An apparently identical spectrum for this peroxy adduct has been found with Halothane ($CClBrH–CF_3$) and chloroform, although the relative yields are smaller.

Recent studies have demonstrated that spin-trap adducts can also be obtained from other halogenoalkanes: $CHCl_3$, $CHBr_3$, CHI_3, CH_2Br_2, Halothane, and dibromoethane (Tomasi *et al.* 1983*a*, *b*, 1984, 1985). In such experiments the concentration of O_2 is a critical feature (for Halothane see, for example, Tomasi *et al.* 1983*a*; for a discussion of this in relation to CCl_4 see, for example, Noll & de Groot 1984).

Reactivity of the free-radical intermediates

The chemical reactivity of $CCl_3^{\cdot}$ has been extensively studied in the liquid phase by the classical kinetic techniques of physical organic chemistry (Walling 1957), although most of the reactions so studied are not directly relevant to the biological situation. After the realization that CCl_4 has to undergo a metabolic activation to $CCl_3^{\cdot}$ to exert its full range of hepatotoxic effects, it was natural to consider that the early damaging reactions that occur were a result of the reactivity of the primary metabolite $CCl_3^{\cdot}$. To gain quantitative information about the chemical reactivity of $CCl_3^{\cdot}$ with important biomolecules, we decided in 1973 to approach that task by using the technique of pulse radiolysis (Willson & Slater 1975). During these early studies it was found that when the reactions were conducted under strictly anaerobic conditions the CCl_3 free radical is relatively unreactive; in fact, no detectable reaction could be observed with a range of compounds such as thiols, nucleotides, amino acids, etc. The introducion of small concentrations of O_2 greatly changed the kinetic features (Packer *et al.* 1978) and leads to the formation of the peroxy free radical $CCl_3O_2^{\cdot}$.

As already mentioned, $CCl_3O_2^{\cdot}$ has a much higher chemical reactivity with biomolecules in solution than $CCl_3^{\cdot}$. The rate constants for $CCl_3O_2^{\cdot}$ in such reactions fall generally in the range 10^6–10^9 M^{-1} s^{-1} (Packer *et al.* 1978, 1981). In contrast, the reactions of $CCl_3^{\cdot}$ with such substances were undetectable with the pulse radiolysis system used and have rate constants less than 10^5 M^{-1} s^{-1}. This does not mean that the chemical reactivity of $CCl_3^{\cdot}$ is negligible in relation to cellular damage (far from it!), but that its reactivity is *relatively* much less than for $CCl_3O_2^{\cdot}$. Because $CCl_3O_2^{\cdot}$ reacts much faster with a PUFA (such as arachidonate) than does $CCl_3^{\cdot}$ it has been suggested that lipid peroxidation is preferentially initiated in the endoplasmic reticulum by $CCl_3O_2^{\cdot}$ rather than $CCl_3^{\cdot}$ (Slater 1982). Conversely, $CCl_3^{\cdot}$ may be responsible for most of the covalent binding detected in liver after exposure to $^{14}CCl_4$, as it is probable that any covalent binding of $CCl_3O_2^{\cdot}$ would be unstable during experimental work-up procedures (Slater 1982).

Finally, in this short account of the reactivity of $CCl_3^{\cdot}$ and $CCl_3O_2^{\cdot}$ it is interesting to note the study by Packer *et al.* (1981), which demonstrated the important influence of the chlorine substituents on chemical reactivity: the reactivity of the free radicals decreases in the order $CCl_3O_2^{\cdot} > CHCl_2O_2^{\cdot} > CH_2ClO_2^{\cdot} > CH_3O_2^{\cdot}$.

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Locus of formation of $CCl_3^{\cdot}$ in liver endoplasmic reticulum

The metabolic activation of CCl_4 in liver endoplasmic reticulum probably occurs through a process of dissociative electron capture (Gregory 1966):

$$CCl_4 + e^- \rightarrow CCl_4^{-\cdot} \rightarrow CCl_3^{\cdot} + Cl^-.$$

In principle, the electron could be supplied directly by the NADPH–cytochrome-P_{450} system (for example, via the NADPH–flavoprotein or cytochrome P_{450}), or indirectly by donation from secondary reductants, such as the superoxide anion radical or an iron chelate, which are themselves reduced by the primary NADPH–cytochrome-P_{450} electron-transport chain. Whether direct or indirect, the activation is certainly closely associated with the NADPH–cytochrome-P_{450} system as many studies have demonstrated (for a review see, for example, Slater 1972, 1982).

A possible secondary route of activation that has already been mentioned is through an interaction of O_2^- and CCl_4 in the non-polar environment of the endoplasmic reticulum. It is known from the work of Sawyer and his colleagues that O_2^- can reduce CCl_4 in aprotic media (Roberts & Sawyer 1981). This pathway, if it occurs at all in the biological situation, would be aided by the destructive effect of CCl_4 activation on cytochrome P_{450} (Glende 1972), thereby encouraging electron outflow to O_2. In liver microsomal suspensions, however, the activation of lipid peroxidation by CCl_4 is not significantly diminished by the addition of superoxide dismutase (O. P. Sharma, K. H. Cheeseman & T. F. Slater 1985, unpublished results). The latter point, together with the effects of several free-radical scavengers on the microsomal lipid peroxidation which is stimulated by CCl_4, are illustrated in table 1.

Table 1. Effects of superoxide dismutase (SDM) and other substances on CCl_4-stimulated lipid peroxidation in rat-liver microsomes[a]

addition	concentration/μM or units	percentage inhibition	EC_{50}/μM[b]
SDM	1000 units	15	—
urate	200	2	—
	500	10	—
indomethacin	20	0	—
	50	12	—
propyl gallate	—	—	2.0
promethazine	—	—	0.5
metiazinic acid	—	—	33
nafazatrom	—	—	16

[a] Data from Sharma *et al.* (1985). [b] Concentration producing a 50% inhibition.

The discussion above has briefly considered the interactions of CCl_4 with the NADPH–cytochrome-P_{450} electron-transport chain; another feature of CCl_4 activation to consider, however, is the location of activation among the different regions of the liver lobules. Administration of CCl_4 *per os*, by inhalation, or by injection into the peritoneum produces necrosis that is essentially centrilobular in nature. It is reasonable to assume that a major contribution to this lobular location of injury is the distribution of the NADPH–cytochrome-P_{450} system itself. It is known (Gooding *et al.* 1978) that cytochrome P_{450} is more concentrated in the centrilobular regions of rat liver than in the periportal regions. However, other factors may

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contribute significantly: for example, the lobular gradient of O_2 (Ji *et al.* 1982), which may ensure the optimal conditions for lipid peroxidation (Noll & de Groot 1984); and the lobular distribution of protective mechanisms, about which little is known, although glutathione is preferentially distributed periportally (Smith *et al.* 1979).

In connection with the metabolic activation of CCl_4 in tissues other than liver, it is known that the NADPH–flavoprotein and cytochrome P_{450} are widely distributed, even if the amounts of P_{450} tissue may be small in many tissues (Benedetto *et al.* 1981). Covalent binding of $^{14}CCl_4$, and adduct formation with the spin trap phenylbutyl nitrone (PBN) *in vitro* generally were correlated with the tissue distribution of P_{450} (Benedetto *et al.* 1981). Studies *in vivo* with PBN (A. Tomasi & T. F. Slater, unpublished results) give broadly similar results. Because cytochrome P_{450} is known to be located in specific cell types in various non-hepatic tissues (such as lung), it is probable that the overall measures of covalent binding, spin-adduct formation, stimulation of lipid peroxidation, etc., expressed per gram wet mass of tissue, hide much greater extents of activation and damage in specific cell types.

Damage to the plasma membrane

Most studies on the damaging actions of CCl_4 on isolated liver cell membranes have been on microsomes, for obvious reasons. The earliest damage *in vivo* that is morphologically evident is to the endoplasmic reticulum (Oberling & Rouiller 1956) and the NADPH–cytochrome-P_{450} system is firmly associated with this intracellular membrane function. Studies with isolated hepatocytes, however, have demonstrated relatively early damage to the plasma membrane when exposed to CCl_4 or Halothane (Perrissoud *et al.* 1981; Jewell *et al.* 1982; Tomasi *et al.* 1983*a*); morphologically this damage is evident as substantial blebbing. It is thus of interest to consider the possibility that CCl_4 is activated by the plasma membrane.

We have isolated highly purified plasma membrane subfractions from rat liver, by using modifications of the method of Wisher & Evans (1975), and have studied the effects of CCl_4 in relation to covalent binding, spin trapping and stimulation of lipid peroxidation. No evidence was found (table 2; Le Page *et al.* 1985) for any significant activation of CCl_4 by the highly

Table 2. Rat liver plasma membranes and activation of CCl_4 compared with data obtained from rat-liver microsomal suspensions.[a] Also shown are some results obtained after exposing normal rat hepatocytes and Novikoff tumour cells to γ-radiation

	microsomes (percentage)	plasma membrane (percentage)
cytochrome P_{450}	100	12
NADPH–cytochrome *c* reductase	100	20
$C_{20:4}$ (percentage total fatty acids)	29	26
NADPH–CCl_4-stimulated lipid peroxidation	100	4
$^{14}CCl_4$ covalent binding	100	3
ascorbate–Fe^{2+}-stimulated lipid peroxidation	100	114
lipid peroxidation stimulated by γ-irradiation	100	73
Novikoff cells against isolated normal hepatocytes as 100%:	normal hepatocytes	Novikoff cells
lipid peroxidation stimulated by γ-irradiation	100	2

[a] Values from Le Page *et al.* (1985).

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purified sinusoidal membrane subfraction; less extensive data for the lateral membrane and canalicular membrane subfractions also point to the same conclusion. In relation to the canalicular membrane data it is known from studies *in vivo* that bile flow is not substantially altered in the early stages of CCl_4 intoxication (Delaney & Slater 1971).

We can conclude from these results that the liver plasma membrane does not significantly metabolize CCl_4 to the $CCl_3^{\bullet}$ free radical. In consequence, the early changes seen in the plasma membrane of isolated hepatocytes may reflect secondary consequences of metabolic activation in the endoplasmic reticulum or, less likely in our view, artefactual perturbations in the plasma membrane of the isolated hepatocyte resulting from the proteolytic method of preparation.

Reactions of $CCl_3^{\bullet}$ and $CCl_3O_2^{\bullet}$

A reactive oxidizing species such as $CCl_3O_2^{\bullet}$ (and, to a lesser extent, $CCl_3^{\bullet}$) can be expected to interact in damaging ways with a variety of substances in the local environment around the locus of metabolic activation (Slater 1984*a*). For example, primary consequences of the metabolic activation of CCl_4 can be expected to be (i) oxidation of thiol groups which may be essential for enzyme activity; (ii) covalent binding to lipid protein, nucleotides, haem, etc., that may greatly change or even destroy biochemical activity. An example is the destruction of NADPH in the liver *in vitro*, during the early phase of CCl_4-induced liver injury (Slater *et al.* 1964); and (iii) by initiating lipid peroxidation which can result in membrane disturbances due to loss of PUFA, cross-linking and production of reactive products.

The destruction of NADPH referred to above can also be studied *in vitro* by using isolated hepatocytes; figure 1 gives some corresponding data with the powerful hepatotoxic agent

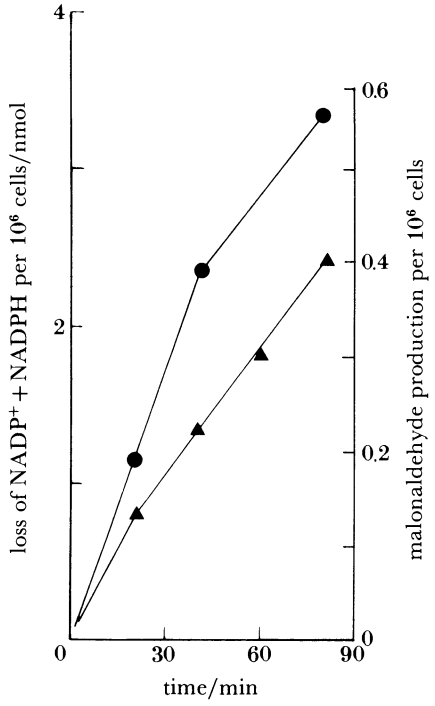

Figure 1. Effect of incubating normal isolated rat hepatocytes with CCl_3Br; ●, loss of $NADP^+ + NADPH$; ▲, production of malonaldehyde (arbitrary absorbance units at A_{535}). Data from R. Scott & T. F. Slater, unpublished results.

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CCl_3Br. It was found (R. Scott & T. F. Slater, unpublished results) that the loss of $NADP^+$ and NADPH during incubation of rat hepatocytes with CCl_3Br correlated with the amount of malonaldehyde-like material produced. CCl_3Br can also be used to study reactions of $CCl_3^{\cdot}$ in solution because CCl_3Br is photochemically degraded to $CCl_3^{\cdot}$ and other products. For example, exposure of a mixture of CCl_3Br and the spin trap PBN to a wide-spectrum mercury lamp results in the appearance of the PBN–CCl_3 adduct, which can be readily detected by e.s.r. (M. Davies, K. A. K. Lott & T. F. Slater 1985, unpublished results). An interesting interaction of $CCl_3^{\cdot}$ with membrane PUFA has recently been discussed by Link *et al.* (1984). In this interaction, the addition of $CCl_3^{\cdot}$ across a double bond is followed by cross-linking with neighbouring fatty-acid chains. If this were of appreciable extent then it could result in a decreased membrane fluidity, with associated consequences for membrane function. In this context it is worth noting that lipid peroxidation is known to decrease microsomal membrane fluidity (Dobretsov *et al.* 1977; Slater 1979). It can be seen from the discussion earlier that CCl_4 can produce a variety of damaging effects on liver cells. In consequence, we believe that the liver damage produced by CCl_4 is multicausal in origin (Slater 1982), and that the relative contributions of such important features as covalent binding and lipid peroxidation to the overall cellular perturbation will vary somewhat with the particular experimental system under study.

Lipid peroxidation

Changes consistent with a stimulation of lipid peroxidation can be detected in liver *in vivo* very shortly after administering CCl_4 to a rat (Rao & Recknagel 1969). With isolated hepatocytes a marked lipid peroxidation can be detected on adding CCl_4 to the incubating medium (Poli *et al.* 1979), without a significant lag phase and long before the appearance of signs of major cell injury, such as the loss of trypan blue staining and leakage of cytoplasmic enzymes. In cytochemical experiments with isolated hepatocytes incubated with CCl_4 and then stained for products of lipid peroxidation (G. Nöhammer, E. Schauenstein, G. Poli, M. U. Dianzani & T. F. Slater 1985, unpublished results), the increased amount of peroxidation products was evident in almost all cells examined, thereby eliminating the possibility that early increases in lipid peroxidation in isolated hepatocytes are confined to a small percentage of the total cell population and, moreover, a small percentage that is composed of 'dead cells'.

There are interesting species differences in the extents of CCl_4-stimulated lipid peroxidation in liver microsomes. Although it was claimed that mouse-liver microsomes did not readily peroxidize when incubated with CCl_4 (Toranzo *et al.* 1978), this result was contradicted by Lee *et al.* 1982). We also find (Proudfoot *et al.* 1985) good peroxidative activity in mouse (and guinea pig) microsomes in comparison with the rat, but rabbit microsomes are much less active (table 3).

Lipid peroxidation is known to produce a variety of products (Slater 1984*b*), including biologically reactive lipid hydroperoxides and aldehydes such as 4-hydroxy-alkenals (Esterbauer *et al.* 1982; Poli *et al.* 1985). Because these products have much longer half-lives in the biological environment in which they are formed than $CCl_3^{\cdot}$ and $CCl_3O_2^{\cdot}$, they can diffuse for much greater distances, even to extracellular regions. With isolated hepatocytes following incubation with ADP–iron or CCl_4, for example, the suspending medium contains significant amounts of aldehydic products of cellular lipid peroxidation (Poli *et al.* 1985). From such considerations we may understand how a precisely localized metabolic activation in the endoplasmic reticulum can result in metabolic disturbances at considerable distances, because of the biological

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TABLE 3. CCl_4-STIMULATED LIPID PEROXIDATION IN LIVER MICROSOMAL SUSPENSIONS PREPARED FROM DIFFERENT ANIMALS

species	number of experiments	stimulation of lipid peroxidation[a]
rat	6	285 ± 27
mouse	5	277 ± 46
guinea pig	7	269 ± 23
rabbit	3	100 ± 18

[a] Values are picomoles of malonaldehyde per minute per milligram of protein (Proudfoot *et al.* 1985). Mean values are given $\pm$s.e.m.

reactivity and diffusion of products of lipid peroxidation. Figure 2 illustrates this concept (Slater 1976).

Because lipid hydroperoxides can affect the activity of cyclo-oxygenase and of other enzymes of the prostaglandin cascade (Hemler *et al.* 1979), it is of interest to consider whether liver injury due to CCl_4 is associated with disturbances of liver eicosanoid metabolism. Our preliminary studies (S. Hewertson, R. G. McDonald-Gibson, J. Hurst, A. Morgan & T. F. Slater 1985, unpublished results) indicate that CCl_4 administration *in vivo* produces an increase in the thromboxane content of liver (measured as TXB_2), but the cellular origin of this material remains to be investigated (Spolarics *et al.* 1984). The increase in TXB_2, if confirmed, is interesting in view of the suggestion (Kanzaki *et al.* 1979) that thromboxane is the trigger for liver-cell division after partial hepatectomy. It is well known that CCl_4-induced liver necrosis is followed by a regenerative phase.

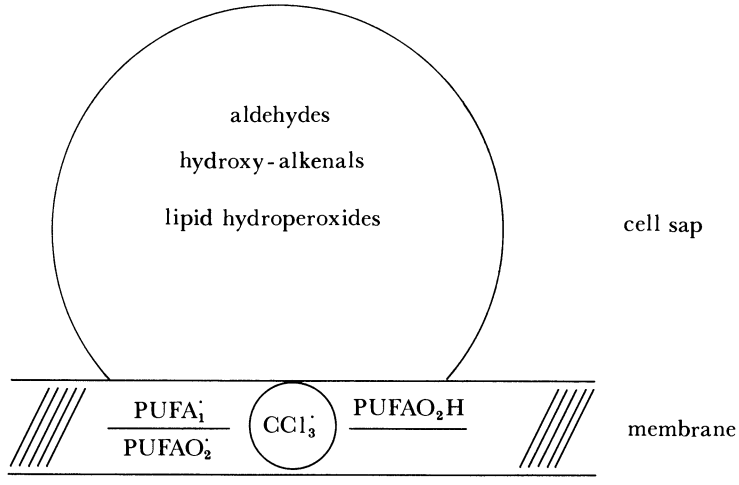

FIGURE 2. A diagrammatic impression of the metabolic activation of CCl_4 to the trichloromethyl radical in the membranes of the liver endoplasmic reticulum. The diffusion of $CCl_3^{\cdot}$ (and even more so, of $CCl_3O_2^{\cdot}$) is shown as restricted to the micro-environment of the site of activation owing to the chemical reactivity of the free-radical intermediate. Other products of the lipid peroxidation, which results from the attack of $CCl_3O_2^{\cdot}$ on membrane polyunsaturated fatty acids, are shown diffusing in the plane of the membrane, or escaping from the membrane into the cytosol. Modified from Slater (1976).

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Protection

Protection against the type of cell injury produced by CCl_4 can be achieved, at least in part, in many ways (for a review see, for example, Slater 1978), including the efficient scavenging of the primary reactive free radicals.

Because of the restricted diffusion and short lifetimes of reactive free radicals such as $CCl_3O_2^{\cdot}$ (and $CCl_3^{\cdot}$), it is evident that effective scavenging of such species must satisfy a number of demanding criteria (Slater 1981); the scavenger must penetrate to the precise intracellular locus of metabolic activation; it must achieve a local concentration sufficient to compete successfully with neighbouring biomolecules that would otherwise be 'damaged' by the attack of $CCl_3^{\cdot}$ or $CCl_3O_2^{\cdot}$; it must reach the critical zones of metabolic activation in time to prevent seriously damaging secondary radical and non-radical processes from occurring; and its own cytotoxic effects must be acceptably low. However, because most free-radical scavengers have many other actions *in vivo* that may significantly affect the development of the injury under study, then, as pointed out elsewhere (Slater 1984*a*), even where a substance has an established scavenging activity, and is present *in vivo* in concentrations appropriate for effective scavenging, it cannot be assumed that this feature is the only or even the major mechanism by which it exerts protective functions *in vivo*.

Liver tumours

Repeated doses of CCl_4 produce cirrhosis and may produce tumours of the liver; CCl_4 is not especially effective as a liver carcinogen (for a literature review see, for example, World Health Organization 1979), and is essentially unreactive in modified Ames tests for mutagenicity (World Health Organization 1979).

Although CCl_4 stimulates lipid peroxidation in acute situations *in vivo* or during incubations *in vitro*, and can produce liver tumours under appropriate conditions, it is of interest that many liver tumours have a much reduced rate of lipid peroxidation (Slater *et al.* 1984; Burton *et al.*

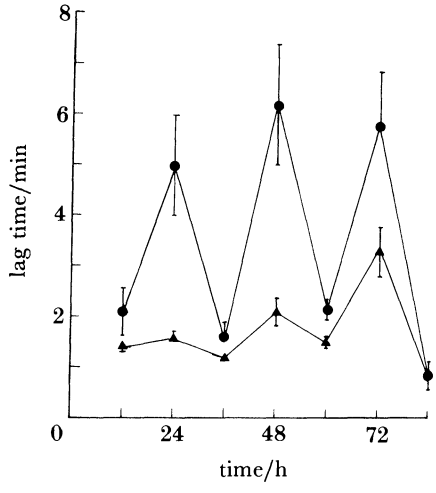

Figure 3. Changes in lipid peroxidation (as measured by the lag time in the NADPH–ADP–Fe^{2+} microsomal system) in regenerating rat liver after partial hepatectomy. The data are from Cheeseman *et al.* (1985). Sham-operated rat-liver samples (▲); regenerating liver samples (●).

1983). Novikoff cells even peroxidize very slowly when γ-irradiated by ^{60}Co (see table 2). A major reason for the slow rate of peroxidation is the increased content of α-tocopherol (Cheeseman *et al.* 1984).

Of course, it is possible that such changes are not directly related to malignant transformation but are perhaps reflections of an increased propensity for cell division. We have investigated this aspect by using regenerating liver after partial hepatectomy as a model of liver cells that are greatly stimulated to divide. In preliminary studies (Cheeseman *et al.* 1985) we have found that there are big changes in the rate of lipid peroxidation at the times of cell division (figure 3).

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Discussion

Catherine Rice-Evans (*Department of Biochemistry, Royal Free Hospital School of Medicine*). Could Professor Slater please explain his observation that hydroxy-alkenals inhibit the aggregation of platelets? Which stimulating agents are involved and what is the mechanism of action?

T. F. Slater. I am unable to give much detail in reply to this question as the results are still preliminary and unpublished. However, I can say that, together with Dr John Hurst in Brunel, we have found that 4-hydroxy-nonenal inhibits platelet aggregation by ADP or arachidonate but has little effect on aggregation stimulated by thrombin, collagen or calcium ionophore. We have also found that 4-hydroxy-nonenal is much more active in these respects than 4-hydroxy-pentenal.

H. Sies (*Institut für Physiologische Chemie I, Universität Düsseldorf, F.R.G.*). The liver seems to be particularly vulnerable at the perivenous end of the liver lobule, given the activation of CCl_4 and the low oxygen tension. Further, as Smith *et al.* (1979) have shown, there are lower contents of GSH in perivenous than periportal cells. Regarding other antioxidants, is the subcellular distribution of vitamin E known?

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T. F. Slater. Not so far as I know. Quite clearly, the intralobular distribution of protective agents and enzymes is one important aspect to consider in relation to the intralobular location of the injury, in addition to the location and activity of associated activating systems (such as the NADPH–P_{450} pathway), and other necessary components such as O_2. These aspects have intrigued us for many years but specific data on vitamin E and related free-radical scavengers are lacking.

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