Carbon tetrachloride, bromotrichloromethane and ethanol acute intoxication

New chemical evidence for lipid peroxidation in rat tissue microsomes

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Second-derivative spectroscopy was used to determine the conjugated-diene shift that measures the extent of the first step of lipid peroxidation after carbon tetrachloride (CCl_4), bromotrichloromethane (BrCCl_3) and ethanol intoxication. The conjugated-diene signal was recorded in the second-derivative spectra as a minimum peak at 233 nm. The use of this method enabled us to show that, under our experimental conditions, CCl_4 - and BrCCl_3 -dependent conjugated-diene formation in rat liver microsomes is not dose-dependent and increases linearly with time up to 3 h. Proportionality was not obtained between the second-derivative-spectroscopy method, and the thiobarbituric acid and difference-spectra methods. In addition, whereas the thiobarbituric acid and difference-spectra methods gave positive results at zero time, second-derivative spectroscopy showed no evidence of formation of conjugated dienes under the same experimental conditions. Intoxication with ethanol was shown by the appearance of the conjugated-diene signal in liver microsomes 24 h after the administration of the toxin. Intoxication with either of the haloalkanes or ethanol did not give rise to any similar peak in lung and brain microsomes. The results obtained are discussed.

The importance of plasma-membrane lipids is well recognized (Menzel, 1976). Because of their high degree of unsaturation, membrane lipids are susceptible to a peroxidative attack that involves free-radical reactions (Slater, 1972). Several pollutants are free radicals, or cause the formation of radicals in exposed tissue, and react with cellular components (Plaa & Witschi, 1976).

The importance of examining changes in structural integrity caused by lipid peroxidation is apparent. A good knowledge of these changes would allow a better understanding of the pathological conditions induced by lipid peroxidation *in vivo* [for reviews, see Slater (1972) and Pryor (1980) and references therein].

The precise role of lipid peroxidation in different drug toxicities has been difficult to evaluate, largely because of lack of sensitive methods for the measurement of lipid peroxidation in experimental models.

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The occurrence of lipid peroxidation can be detected and measured directly by (i) titration with iodide of peroxide groups or (ii) by detection of the absorbance at approx. 233 nm due to the conjugated-diene function, or (iii) indirectly by measuring the breakdown products of lipid peroxides such as malonaldehyde, ethane, pentane and fluorescent chromophores produced by the further reaction of malonaldehyde with nitrogenous materials.

All these methods have been reviewed and criticized (Slater, 1972; Bus & Gibson, 1979; Pryor, 1980; Logani & Davies, 1980).

In the direct spectrophotometric estimation of the conjugated dienes, which may be considered the most suitable method for evaluating the damage at zero time, it is difficult (i) to determine the precise wavelength of the maximum absorption and (ii) to measure the height of the peak of the absorbance, which could be used to measure quantitatively the conjugated double bond, because of the rather imprecise shoulder of the diene absorption on the strong absorption peak due to the polyunsaturated fatty acid(s) itself.

Different methods have been developed to avoid these problems (Recknagel & Ghoshal, 1966; Thomas *et al.*, 1968; Klein, 1970; Pryor, 1980).

Recently we have proposed a simple and reliable method for the direct detection of conjugated dienes formed *in vivo* in polyunsaturated fatty acids (Corongiu & Milia, 1983) based on second-derivative spectroscopy, which is of great advantage whenever the qualitative and quantitative evaluation of normal spectra is problematic.

This new method allows the evaluation of the first damage induced by oxidants to unsaturated lipids with better accuracy, reliability and reproducibility than other methods that are used to detect the initial steps of lipid peroxidation.

Our purpose is to see whether this method is suitable for the determination of the diene-conjugation signal in microsomes of animals intoxicated with carbon tetrachloride (CCl_4), bromotrichloromethane (BrCCl_3) and ethanol, and to compare this method with two of the most frequently used direct and indirect tests for the detection of lipoperoxidative process, the so-called 'difference-spectra method' (Recknagel & Ghoshal, 1966), used for detecting the diene-conjugation signal, and the thiobarbituric acid reaction for malonaldehyde determination (Sinnhuber *et al.*, 1958), as modified by Ohkawa *et al.* (1979), which permits the detection of malonaldehyde at zero time without exogenous induction.

In addition, because the precise role of lipid peroxidation in different drug toxicities has been difficult to evaluate because of lack of sensitive methods for the measurement of lipid peroxidation in experimental models, we have chosen these three different drugs as models for evaluating qualitative and quantitative differences. In fact, BrCCl_3 , although over 10-fold more toxic than CCl_4 (Slater, 1972), is considered to induce hepatic damage through the same mechanisms as CCl_4 ; ethanol's role as a lipid-peroxidation-inducing drug is still disputed (Di Luzio & Hartman, 1967; Hashimoto & Recknagel, 1968).

Materials and methods

Chemicals

CCl_4 , BrCCl_3 , chloroform, methanol, ethanol and cyclohexane, all spectrophotometric grade, were purchased from Merck, Darmstadt, Germany. 2-Thiobarbituric acid [5,5-diethyldihydro-2-thioxopyrimidine-4,6(1*H*,5*H*)-dione] was obtained from Aldrich Europe, Beerse, Belgium, and 1,1,3,3-tetraethoxypropane from Schuchard, Munich, Germany. All other chemicals used were of analytical grade.

Animal treatment

Male Sprague-Dawley rats (Charles River) weighing 150–200 g were fed a standard diet (Ditta Piccioni, Brescia, Italy) and water *ad libitum*. The food was removed 12 h before treatment.

CCl_4 (13 or 26 mmol/kg body wt.) or BrCCl_3 (13 mmol/kg body wt.) dissolved in mineral oil (5 ml/kg body wt.) were administered by stomach tube at various times before the animals were killed. Control rats received mineral oil alone (5 ml/kg body wt.).

A single dose of ethanol [1.57 ml of 50% (w/v) ethanol/100 g body wt.] was administered by stomach tube. Rats serving as controls received glucose as an aq. 50% (w/v) solution at a dose of 2.4 ml/100 g body wt. Rats were killed 24 h after ethanol or glucose administration.

Preparation of homogenates

After treatment the rats were anaesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal) (50 mg/kg body wt.) dissolved in heparinized 0.9% NaCl solution, and the livers and lungs were perfused *in situ* with ice-cold iso-osmotic NaCl.

The organs were quickly removed and washed in ice-cold iso-osmotic NaCl. The livers were passed through a pre-cooled tissue press. Liver pulp free from connective tissue, lung and brain were homogenized either with a Potter-Elvehjem homogenizer fitted with a Teflon pestle or with a Polytron homogenizer (Kinematica G.m.b.H., Lucerne, Switzerland). Livers were homogenized in 9 vol. of ice-cold 0.25 M-sucrose containing 5 mM-EDTA, whereas lung and brain were homogenized in 2 vol. of the same medium. Microsomal preparations for the thiobarbituric acid test were obtained as described above, except that EDTA was omitted.

Isolation of microsomes

The homogenates were centrifuged at 14 500 g for 15 min at 0–4°C. From the postmitochondrial supernatant the microsomes were spun down at 105 000 g for 60 min.

The microsomal pellets thus obtained were rinsed in ice-cold 0.15 M-KCl and, if not immediately used, stored in a deep-freezer.

Extraction of lipids

The method used was essentially that of Folch *et al.* (1957). The lipid extract was used for the quantitative determination of total lipids (Chiang *et al.*, 1957) and for the diene-conjugation absorption studies (Recknagel & Ghoshal, 1966; Corongiu & Milia, 1983).

Determination of the diene-conjugation signal

The lipid/chloroform solutions were placed in glass test tubes with ground-glass stoppers. The solvent was extracted under vacuum at room temperature. The lipids were then dissolved in cyclohexane in order to obtain a lipid concentration of 100 µg/ml.

The lipid solution was then immediately scanned from 300 to 220 nm and the absorbance and the second-derivative spectrum were recorded.

Before the scanning operation a background-correction-memorized scan between Suprasil cells containing cyclohexane was performed in order to

avoid spectral differences between sample and reference cell.

The results of the second-derivative spectra were expressed in arbitrary units as $d^2A/d\lambda^2$ per 100 µg of lipid, where $d^2A/d\lambda^2$ represents the height of the minimum peak at 233 nm in the second-derivative spectrum, expressed as absorbance (*A*) units.

Thiobarbituric acid test

Microsomal fractions were resuspended in 1.15% KCl so as to contain approx. 1 mg of protein in 0.1 ml of suspension. The protein content was

Fig. 1. U.v. (a) and second-derivative (b) spectra of lipid extracted from liver microsomes of rats I, Untreated; II, 5 min after intoxication with CCl₄ (13 mmol/kg body wt.); III, 24 h after administration of ethanol [1.57 ml of 50% (w/v) ethanol/100 g body wt.]. The microsomal pellet obtained from 1 g of fresh tissue was resuspended in 8 ml of methanol, to which 16 ml of chloroform were added and mixed. The tubes were left for 1 h at room temperature in the dark, then 8 ml of distilled water was added. After mixing, the tubes were stored overnight at 4°C. After centrifugation (600 g for 10 min) the chloroform phase was used for the quantitative determination of total lipids and for diene-conjugation absorption studies. Microsomal lipids (100 µg/ml in cyclohexane) were scanned from 300 to 220 nm. The minimum peaks in the second-derivative spectra represent the relation $d^2A/d\lambda^2$, where *A* is the absorbance and λ the wavelength and are measured in arbitrary units as the difference between the absorbances from the shoulder and the minimum at the characteristic wavelength. In the second-derivative spectra of II and III is evident a minimum peak that characterizes the conjugated-diene shift at 233 nm (arrow).

determined by the Lowry *et al.* (1951) method, with bovine serum albumin as standard.

The thiobarbituric acid reaction was performed as described by Ohkawa *et al.* (1979). Tetraethoxypropane was used as internal standard and the level of lipid peroxides was expressed as nmol of malonaldehyde/mg of microsomal protein.

Statistical analysis was performed by using Student's *t* test with a preset probability level of $P < 0.05$.

Results

Fig. 1 shows the u.v. and the second-derivative spectrum of microsomal lipids obtained from liver of untreated and treated rats.

We have recently shown (Corongiu & Milia, 1983) that the second-derivative spectrum is useful to demonstrate the presence, with a minimum peak at 233 nm, of the diene-conjugation signal. As Fig. 1 shows, in liver lipid extracts from normal animals this signal is absent, although the minimum peak at 233 nm is evident 5 min after treatment of the rats with CCl_4 at the dose of 13 mmol/kg body wt., and 24 h after administration of ethanol.

Treatment of animals with paraffin or glucose does give rise to a signal in the second-derivative spectrum (results not shown).

Although we used great care, we have remarked that, under our experimental conditions, 'artefactual' peroxidation may occur in lipid extracted from microsomes of normal rats obtained after homogenization with the Potter-Elvehjem homogenizer, although this phenomenon is negligible in those obtained after homogenization with the Polytron. The Polytron was consequently used for all microsomal preparations.

Results obtained with 100 $\mu\text{g}/\text{ml}$ and 200 $\mu\text{g}/\text{ml}$ concentrations of lipid extracted from liver microsomes of rats after 1 h intoxication with CCl_4 (13 mmol/kg body wt.) show that the height of the second-derivative minimum peak at 233 nm is linearly dependent upon the concentration of the peroxidized fatty acid(s) itself (results not shown).

As Fig. 2 shows, in rats treated with halogenoalkanes (at the doses of 13 and 26 mmol of CCl_4/kg body wt. and 13 mmol of BrCCl_3/kg body wt.), the height of the peak in the second-derivative spectrum increases linearly up to 3 h after poisoning, whereas when the extent of lipid peroxidation is measured by the thiobarbituric acid test at zero time (Ohkawa *et al.*, 1979), no significant difference was found. In addition, the height of the peak obtained by constructing the difference spectra between microsomal lipids from normal and intoxicated rats does not increase linearly with time and is not proportional to the height of the peaks obtained with the second-derivative-spectroscopy method. Further-

Fig. 2. Time-dependent relationship between the measurement of the extent of lipid peroxidation obtained by second-derivative spectrum (curve I), by thiobarbituric acid test at zero time (curve II) and with difference spectra (curve III) of rat liver microsomal lipids obtained from CCl_4 - (13 mmol/kg body wt.; ○, □, △) and BrCCl_3 - (13 mmol/kg body wt.; ●, ■, ▲) treated animals.

Results are expressed as means $\pm$ S.E.M. for five animals. Curve I: experimental conditions and A units are given in the legend of Fig. 1. Curve II: microsomal fractions were obtained as described in the Materials and methods section, except for EDTA, which was omitted in the preparative solution. Microsomes were suspended in 1.15% KCl so as to contain approx. 1 mg of protein/0.1 ml of suspension. The reaction mixture, which contained 0.1 ml of sample, 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% (v/v) acetic acid adjusted to pH 3.5 with NaOH, 1.5 ml of aq. 0.8% thiobarbituric acid and 0.7 ml of distilled water, was heated in a boiling-water bath for 60 min. After cooling, 1 ml of distilled water and 5 ml of a mixture of butan-1-ol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 4000 *g* for 10 min the organic phase was taken and its A_{532} was measured. The concentration of lipid peroxides is expressed as nmol of malonaldehyde/20 mg of protein, which was calculated from the A_{532} with tetraethoxypropane as internal standard. Curve III: experimental conditions are given in the legend to Fig. 1. The u.v. spectrum of extracted lipids was determined from 300 to 220 nm. Absorption readings were normalized on the basis of lipid concentration and referred to the same concentrations of lipids obtained from treated rats (100 $\mu\text{g}/\text{ml}$). Difference spectra between the experimental samples and controls were calculated and related to the standard concentration and expressed as ΔA_{233} .

more, the peak of the difference spectra constructed under our experimental conditions never had a maximum at 233 nm; instead a maximum at about 240–242 nm was obtained.

At longer times, the proportionality shown by the signal obtained with the second-derivative-spectroscopy method disappears and the extent of lipid peroxidation as measured by our proposed method seems to be independent both of the dose of the halogenoalkane administered and of the length of the time of the treatment.

In lipid extracted from lung and brain microsomes of animals treated with halogenoalkanes or ethanol there is no evidence of diene signal (results not shown).

Discussion

The control of lipid peroxidation is of special significance in biology because of its particular importance in relation to membrane damage (Slater, 1976).

The toxicity of CCl₄ was first associated with the formation of the trichloromethyl radical (CCl₃·) by Butler (1961).

Packer *et al.* (1978) have demonstrated that in the presence of O₂, CCl₃· readily interacts with it to form CCl₃O₂·.

Indirect evidence suggests that the metabolism of BrCCl₃ follows pathways similar to those of CCl₄ metabolism (Burdino *et al.*, 1973; Bini *et al.*, 1975). In addition, BrCCl₃ is estimated to be over 10-fold more toxic than CCl₄ (Slater, 1972).

The diene-conjugated species have been shown to have a very transient existence, and efforts to correlate the extent of formation of reaction products of halomethane species with damage to cellular components have encountered numerous difficulties because '... many of the macromolecular products formed in the damage producing processes are subject to further alterations and/or are rapidly removed by ongoing endogenous repair processes.' (Reynolds & Moslen, 1980).

Therefore the magnitude of their formation is difficult to determine under conditions *in vivo* and, by means of the commonly used analytical methods, under conditions *in vitro*.

Most studies on lipid peroxidation have been done under conditions *in vitro* with rat liver microsomes, the thiobarbituric acid reaction for malonaldehyde estimation or the diene-conjugation measurement being used.

We have shown that the measurement of the second-derivative signal at 233 nm, corresponding to the diene-conjugated species, makes possible, at least to a certain extent, a linear correlation between the extent of formation of the first product of the reaction of the halogenomethane species used and the extension of the damage with time. In addition, under our experimental conditions, the magnitude of the signal and the time over which this signal

increases proportionally (although BrCCl₃ is considered 10 times more toxic than CCl₄) seem to be dose- and species-independent.

This seems clearly related to the classical free-radical-mediated chain reaction in which the first stimulus regulates all the steps of the reaction.

Slater & Sawyer (1970) found that stimulation of lipid peroxidation *in vitro* by a series of halomethanes was proportional to the bond-dissociation energies required for free-radical production. The bond-dissociation energies refer to the formation of CCl₃·, which was found by Packer *et al.* (1978) not to be chemically very reactive, except with dioxygen.

CCl₃· is produced more easily from CCl₄ than from BrCCl₃, and generally the damaging effects that occur after administration *in vivo* of BrCCl₃ are more serious than those obtained after CCl₄ intoxication (Slater, 1972; Congiu *et al.*, 1972; Burdino *et al.*, 1973).

The microsomal NADPH-cytochrome *P*-450 electron-transport chain has been implicated in the metabolic activation of halomethanes, the precise locus of activation being the object of long scientific debate (McLean, 1967; Slater, 1972; Glende, 1972; Ugazio *et al.*, 1973; Slater, 1982).

The activation of the NADPH-flavoprotein, which may initiate peroxidation by one-electron donation and which acts as the reducing source for cytochrome *P*-450 (Svingen *et al.*, 1979), is decreased after activation of either CCl₄ or BrCCl₃. Consequently we suppose that, after the formation of the same free-radical species *in vivo*, the resulting damaging effects, which are related to conjugated-diene formation, are, to a certain extent, independent of the amount of free radical generated, but depend on the chemical reactivity of the species considered. We think that, *in vivo*, correct quantitative differences in CCl₄- or BrCCl₃-induced lipid peroxidation can be obtained only by studying the activation reactions at very early times and/or by using very small amounts of the toxins.

The time-dependent signal corresponding to the extent of the injury is demonstrated only when it is measured with the second-derivative spectrum. In fact its determination with the difference-spectra method (Recknagel & Ghoshal, 1966) does not linearly correlate with the time-related extent of the injury. The method of Recknagel & Ghoshal (1966), although suitable only for qualitative evaluation of the damage (Logani & Davies, 1980), must be remembered as a very remarkable and intelligent effort to demonstrate directly the existence of a chemical modification following a damaging reaction.

The thiobarbituric acid test for malonaldehyde production at zero time, performed as described by Ohkawa *et al.* (1979), was completely unable to demonstrate the chemical modification subsequent

to the injury. In addition, whereas the method of Recknagel & Ghoshal (1966) makes qualitative evaluations of the damage possible, the method of Ohkawa *et al.* (1979) does not even reveal quantitative differences between control and treated rats.

With other tissues, such as brain and lung, there was no evidence of halomethane-stimulated lipid peroxidation.

In fact the extent of the injury produced by halomethanes in various tissues of the rat probably depends on a considerable number of cellular factors. In particular it has been reported that, in the brain and lung tissues, the contents of cytochrome P-450 and arachidonic acid are considerably lower than those in the liver (Benedetto *et al.*, 1981).

The biochemical mechanisms of ethanol toxicity is a matter of a very old scientific debate between different research groups.

Slater (1972) has suggested the conversion of ethanol into an ethoxy radical ($C_2H_5O^\bullet$), whereas Reitz (1975) proposed that the microsomal enzyme system was implicated as a possible mechanism for producing lipid peroxides after chronic ingestion of ethanol.

The possible occurrence of lipid peroxidation in liver of animals intoxicated with ethanol was first suggested by Di Luzio (1963). Later Kalish & Di Luzio (1966) and Di Luzio & Hartman (1967), by measuring the diene-conjugation spectra, reported an increased peroxide concentration in the liver of intoxicated animals; Hashimoto & Recknagel (1968), however, were not able to confirm these results.

More recently Köster *et al.* (1977) have correlated ethanol intoxication and lipid peroxidation by measuring ethane evolution during the intoxication.

In our experiments the signal at 233 nm, which characterizes in the second-derivative spectra the diene-conjugation shift, is clearly evident in rat liver microsomes 24 h after acute ethanol intoxication.

In accordance with the hypothesis of Slater (1972), we suggest that the ethanol may act also as a free-radical hepatotoxin. Our findings provide further and direct evidence to support the reports of different authors (Kalish & Di Luzio, 1966; Di Luzio & Hartman, 1967; MacDonald, 1973; Köster *et al.*, 1977), who have been able to demonstrate the presence of molecules derived from ethanol-induced lipid peroxidation.

Our findings show also that the amount of conjugated dienes in ethanol-treated rats is lower than in those given CCl_4 . This is in accordance with the findings of Köster *et al.* (1977), who obtained similar results in rats by measuring the quantities of ethane produced as a result of administering the same agent. The amounts of conjugated dienes formed 24 h after ethanol administration parallels those obtained 5 min after CCl_4 intoxication.

These results are in accordance with the hypothesis that we have previously formulated. In fact the chemical reactivity of $C_2H_5O^\bullet$ is much lower compared with that of $CCl_3O_2^\bullet$ (Slater, 1976), consequently the damaging effects exerted by CCl_4 and $BrCCl_3$ are higher than those exerted by ethanol.

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