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THE MEASUREMENT OF LIPID PEROXIDATION IN ISOLATED HEPATOCYTES

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Abstract—Different techniques for the measurement of lipid peroxidation in isolated hepatocytes have been compared. Measurements of ethane production, chemiluminescence and fluorescent products correlated extremely well with those of malondialdehyde formation. Of the five different techniques studied, measurements of ethane production and chemiluminescence were found to be the most sensitive indices of lipid peroxidation. Incubation of hepatocytes for up to 4 hr in the presence of ethylmorphine and aminopyrine, at concentrations known to stimulate H_2O_2 production, completely failed to increase the amount of chemiluminescence, malondialdehyde or ethane produced in these cells, indicating that the drug-stimulated production of H_2O_2 did not lead to an increased rate of lipid peroxidation in liver cells under the experimental conditions employed. The relationship between lipid peroxidation, as measured by chemiluminescence and ethane production, and the cytotoxic effects of bromobenzene and carbon tetrachloride has also been studied. The results obtained further indicate that lipid peroxidation is an important event in carbon tetrachloride hepatotoxicity, but that it appears to be only a subsequent event in bromobenzene toxicity, possibly occurring only as a result of glutathione depletion and cell death.

The peroxidation of polyunsaturated fatty acids present in the membrane lipids is considered to be a basic mechanism of toxicity for a wide variety of chemicals including carbon tetrachloride and ethanol [1–4], but its role in the toxic effects of other compounds, including the herbicide paraquat and the anthracycline drugs adriamycin and daunomycin, remains a highly controversial matter [4–8]. The biochemical consequences of lipid perodixation are well-known and include membrane damage, enzyme inhibition, release of lysosomal enzymes and protein–protein cross-linking [4, 9, 10]. However, 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 complex experimental models, such as isolated perfused organs and freshly isolated cells. For example, the commonly used thiobarbituric acid assay of malondialdehyde production may not be entirely suitable for assessing the extent of lipid peroxidation in intact organs and cells, because malondialdehyde is metabolized at a substantial rate by the mitochondria [11, 12] and also reacts with many other cellular components [13]. Its measurement may therefore give an underestimation of the true level of lipid peroxidation in intact cells.

In the last few years two new methods for determining lipid peroxidation, namely hydrocarbon gas evolution and chemiluminescence measurement, have emerged. The evolution of the hydrocarbon gases ethane and pentane has recently been proposed as a reliable and senstive index of lipid peroxidation both *in vivo* [14, 15] and in experimental model systems, such as the isolated perfused rat liver [16] and tissue homogenates [17]. Chemiluminescence production in hepatic microsomes during either NADPH- or ascorbate-induced lipid peroxidation has also recently been shown to correlate closely with malondialdehyde formation [18] and its measurement is now being extensively used in the study of free-radical reactions, connected with lipid peroxidation, in isolated perfused organs [19–21]. In the present study, these techniques have been adapted for use with suspensions of freshly isolated hepatocytes, which in recent years have proved to be an extremely useful tool in studies on drug hepatotoxicity [11, 22, 23]. The value and relative merits of these and other more established methods for determining lipid peroxidation in isolated cells have been assessed and the most sensitive techniques have been directly applied to the study of drug-induced lipid peroxidation in isolated liver cells.

MATERIALS AND METHODS

Materials

Collagenase (Grade II) and bovine serum albumin (Fraction V) were obtained from Boehringer Mannheim GmbH, Mannheim, West Germany and the Sigma Chemical Co., St. Louis, MO, U.S.A., respectively. Porapak Q (Mesh size 100–120) was obtained from Waters Associates Inc., Milford, MA, U.S.A. and calibrated gas mixtures of ethane and *n*-pentane were from Scott Environmental Technology Inc., Plumsteadville, PA, U.S.A. Other chemicals were at least of reagent grade and purchased locally. Krebs–Henseleit buffer [24] containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), pH 7.4, was used for the incubations.

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Cell preparation

Isolated hepatocytes were prepared from male Sprague–Dawley rats (200–220 g, fed *ad libitum*) as previously described [24]. The rats were pretreated with phenobarbital (80 mg/kg) for five days prior to use for cell preparation. The yield of each preparation was $2–4 \times 10^8$ cells per liver and immediately after isolation the cells excluded both Trypan Blue and NADH (90–100%). Cell viability was determined during the course of the experiments by the exclusion of Trypan Blue and by the NADH penetration assay [24].

Measurement of lipid peroxidation

The total amount of lipid peroxidation in the isolated hepatocytes was determined by five different methods as follows:

Malondialdehyde production. The hepatocytes were incubated at 37° in rotating round-bottom flasks (50 ml) under a 93.5% O_2/6.5% CO_2 atmosphere at a cell concentration of 10^6 cells/ml in the Krebs–Henseleit buffer, pH 7.4. The amount of malondialdehyde present in 0.5 ml samples of these incubates was determined by the thiobarbituric acid method [25] using an extinction coefficient of $156\ mM^{-1}\ cm^{-1}$ [26]. The results are expressed as nmoles malondialdehyde produced/10^6 cells.

Assay of fluorescent products. The fluorescent products of lipid peroxidation were determined using a procedure based on that of Fletcher *et al.* [27] for the measurement of such products in tissue extracts. Incubations were performed as described above. 1 ml aliquots of incubation mixture were added to 0.2 ml perchloric acid (70% v/v) and the suspension vortex mixed until homogenous. It was then extracted into 2 ml isobutanol by a further 10 sec vortex mixing. Following centrifugation, the organic extract was decanted off and stored on ice until required. The fluorescence intensity of each extract was measured at room temperature in an Aminco–Bowman spectrofluorometer using excitation and emission wavelengths of 360 and 460 nm, respectively. One-mm slits were used in the 3, 4 and 6-slit positions and the detector was adjusted to its highest sensitivity. A 1 μg/ml quinine sulphate solution was used as a standard for fluorescence intensity and wavelength calibration and at a meter multiplier setting of 3, this solution had a relative fluorescent intensity of approx 55 and 110 units when the sensitivity vernier was set at 50 and 100, respectively. The arbitrary fluorescence units used in the figures were derived by multiplying the meter multiplier value by the per cent fluorescence $\times 10^3$ and all values are corrected for variations in the intensity of the light source, as previously described [27].

Measurements of chemiluminescence. The hepatocytes were incubated in rotating flasks exactly as described above but all procedures were performed in the dark. At specific times 5 ml aliquots were transferred from the incubation flasks to dark-adapted plastic scintillation vials (7 ml). The amount of chemiluminescence present in each vial was then determined as counts per minute in Channel 5 of an LKB 1216 RackBeta liquid scintillation counter (LKB Wallac, Turku, Finland) operated in the out-of-coincidence mode [28]. Suitable control vials for the subtraction of thermal background were also measured at each time interval. Each vial was counted for at least 30 sec. The chemiluminescence measurements are expressed as counts/sec or cps.

Ethane production. The evolution of ethane was measured using a procedure based on that of Riely *et al.* [17] except that isolated cells were used instead of tissue homogenates. Isolated hepatocytes (10^6 cells/ml), in 50 ml Krebs–HEPES buffer, pH 7.4, which had been oxygenated prior to use by bubbling carbogen (93.5% O_2/6.5% CO_2) through it for at least 10 min, were incubated with gentle shaking at 37° in Erlenmeyer flasks (total volume 67 ml) fitted with screw-caps and rubber septum inlets (Schott GL 18). 0.2–1.0 ml samples of the head space gas were taken at specific times using a gas-tight pyrogen-free syringe. These samples were analyzed immediately for ethane using a Beckman GL 72-5 gas chromatograph fitted with a flame ionization detector and a glass column, 2 m long and 2 mm in diameter, packed with Porapak Q. The operating conditions were: column temperature, 90°; injection block temperature, 120°; detector temperature, 200°; and nitrogen carrier-gas flow rate of $30\ ml^{-1}$. The retention time of ethane was 1–2 min under these conditions. Ethane standards, prepared by suitable dilution of a calibrated gas mixture in the concentration range 1–100 pmoles ml^{-1}, were also analyzed in each experiment. Ethane production is expressed in pmoles gas produced per 10^6 cells and calculated using an adaptation of the expression given by Wendel and Dumelin [15] as follows:

$$\text{pmoles ethane}/10^6 \text{ cells} = \left[a_i \cdot (V_F - V_C) + \sum_{i=0}^{n} a_i \cdot V_S\right] \cdot V_C^{-1}$$

where, V_F = total volume of the flask (ml); V_C = volume of the cell incubate containing cells at a concentration of 10^6 cells/ml (ml); V_S = total sample volume withdrawn from the head space (ml); i = number of samples withdrawn previously; and a_i = ethane concentration calculated from the gas chromatograph trace (pmoles/ml).

Pentane production. The evolution of *n*-pentane from the isolated cell suspensions was determined in the same manner as described for ethane with the following modifications: (1) The operating conditions of the gas chromatograph were: column temperature, 130°; injection block temperature, 160°; and detector temperature, 200°. The retention time of *n*-pentane was approximately 8 min under these conditions. (2) Pentane is slightly soluble in water, whereas ethane is totally insoluble. This was accounted for in all determinations of *n*-pentane production. In preliminary experiments it was found, by rapidly heating the flasks to 60° following incubation, essentially as in [16], that all the ethane produced during the incubation was evolved into the head space whereas only 49.53 ± 2.08 (mean ± S.E.M. of 4 separate determinations) of the *n*-pentane produced was actually present in the head space at 37°. All the pentane measurements were therefore multiplied by a factor of 2.019 in order to express the results as pmoles pentane produced/10^6 cells.

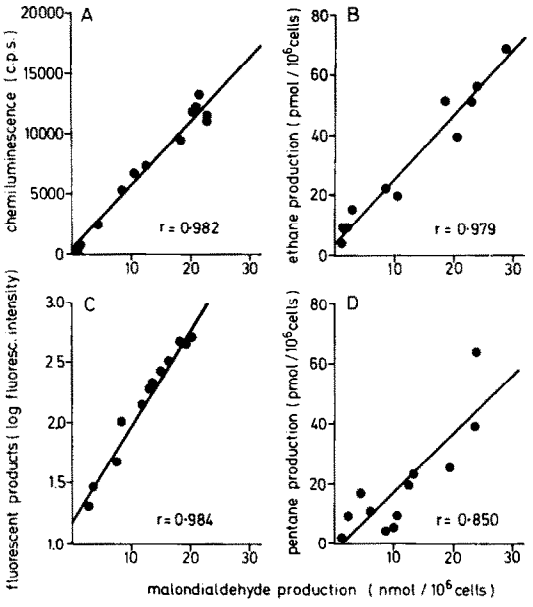

Fig. 1. Correlation of measurements of chemiluminescence, fluorescent products, ethane and pentane production with those of malondialdehyde formation. Five different methods were used to assess the extent of lipid peroxidation in hepatocytes incubated at 37° in the presence of 5 mM carbon tetrachloride at specific intervals between 1 and 4 hr. The results obtained by the four most recently developed methods were then directly correlated with those obtained by the more established thiobarbituric acid assay using linear regression analysis. The results shown are taken from 3 separate experiments. (A) Chemiluminescence vs malondialdehyde production: (B) ethane vs malondialdehyde production; (C) fluorescent products vs malondialdehyde production; and (D) pentane vs malondialdehyde production.

RESULTS

Comparison of the different methods by measuring carbon tetrachloride-stimulated lipid peroxidation in isolated hepatocytes

Five different methods were used to assess the extent of lipid peroxidation in isolated hepatocytes exposed to carbon tetrachloride (5 mM) for 1–4 hr. The results obtained using the four most recently developed methods, namely the assays of fluorescent products, chemiluminescence, ethane and pentane, were then directly correlated with those obtained by the commonly-used thiobarbituric acid assay of malondialdehyde formation (Fig. 1). Measurements of the amount of chemiluminescence or ethane produced by the liver cells correlated extremely well (correlation coefficients = 0.982 and 0.979, respectively) with measurements of the amount of malondialdehyde produced (Fig. 1A, B), indicating that chemiluminescence and ethane production are both directly proportional to malondialdehyde formation in isolated hepatocytes.

Figure 1(C) shows that a close correlation also exists between the formation of fluorescent products, expressed as the log of the fluorescence values, and malondialdehyde production in isolated liver cells (correlation coefficient = 0.984). A much poorer correlation was found, however, between pentane evolution and malondialdehyde formation (Fig. 1D). The correlation coefficient produced from three separate experiments was only 0.850 and possibly reflects varying levels of further metabolism of both pentane and malondialdehyde in intact liver cells.

Although chemiluminescence and ethane production correlated extremely well with malondialdehyde formation after 1–4 hr incubation (Fig. 1A, B), at earlier times a much poorer correlation was found (data not shown). For example, increases in chemiluminescence and ethane production were routinely detected before malondialdehyde formation. This suggested that the thiobarbituric acid assay may be insensitive for the measurement of low levels of lipid peroxidation. It was therefore decided to compare the five different methods at both early (min) and later (hr) times and to express the values obtained in terms of the increase over the zero time level. In this manner it was hoped to determine which method gives the most sensitive and accurate measurement of lipid peroxidation. Hepatocytes (10^6 cells/ml) were incubated at 37° in the presence of carbon tetrachloride (5 mM). Samples were analyzed by the five different methods at 20 min intervals up to 2 hr and then at hourly intervals. Figure 2 shows that ethane production was by far the most sensitive method for measuring carbon tetrachloride-stimulated lipid peroxidation in isolated hepatocytes. A significant increase in ethane production over the zero time level was found after only 20 min incubation (Fig. 2), whereas similar increases were not found in chemiluminescence and pentane production until after 1.5 hr, and at even later times with respect to malondialdehyde and fluorescent product formation (Fig. 2). It is also clear from Fig. 2 that measurements of malondialdehyde, pentane and fluorescent product formation underestimate the true level of carbon

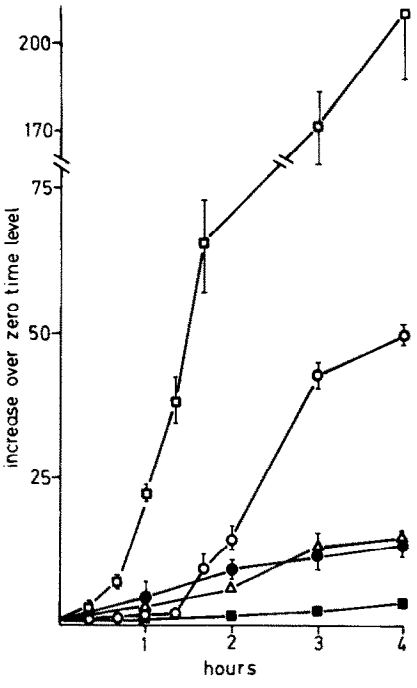

Fig. 2. Comparison of the sensitivity of different assays of lipid peroxidation. Hepatocytes (10^6 cells/ml) were incubated at 37° in the presence of 5 mM carbon tetrachloride. The extent of lipid peroxidation was determined by five different methods at various times and the results expressed in terms of the increase over the zero time level. (●), Malondialdehyde production; (■), fluorescent products; (○), chemiluminescence; (□), ethane production; and (△), pentane production. Results are expressed as the mean values (± S.E.M.) of 4 separate experiments. The zero time values were: 1.56 nmoles malondialdehyde/10^6 cells; 154 arbitrary fluorescence units (fluorescent products); 206 cps (chemiluminescence); 0.3 pmoles ethane/10^6 cells; and 1.67 pmoles pentane/10^6 cells.

tetrachloride-stimulated lipid peroxidation in isolated liver cells. This is probably due to the further metabolism of malondialdehyde and pentane in intact cells and the interaction of malondialdehyde with cellular components, thereby decreasing the total assayable amount. Inability to detect significant increases in malondialdehyde, pentane or fluorescent product formation may therefore not indicate an absence of lipid peroxidation, but may only reflect the insensitivity of these methods.

Studies on the relationship between drug-stimulated H_2O_2 production and lipid peroxidation

During the incubation of hepatocytes with ethylmorphine, aminopyrine and various other drugs, there is an increased rate of hydrogen peroxide (H_2O_2) production [29, 30] and a concomitant release of glutathione disulphide from the liver cells [30, 31]. One of the possible implications of this drug-induced oxidative stress may be an increased rate of lipid peroxidation in the liver cells [30].

In order to test this hypothesis, freshly isolated hepatocytes (10^6 cells/ml) were incubated for up to 4 hr in the presence and absence of ethylmorphine and aminopyrine, at concentrations known to significantly increase H_2O_2 production in liver cells [29–31]. The amount of lipid peroxidation occurring in the isolated hepatocytes was measured at hourly intervals using the two most sensitive methods, namely the assays of chemiluminescence and ethane production (Fig. 2), as well as the more established assay of malondialdehyde formation. Incubation of hepatocytes with 2 mM ethylmorphine or 5 mM aminopyrine has been shown to significantly increase intracellular H_2O_2 production [29–32], but in the present study no significant increase in the overall rate of lipid peroxidation was observed in the presence of either of these drug substrates over the 4 hr period of incubation (Fig. 3). In fact, the presence

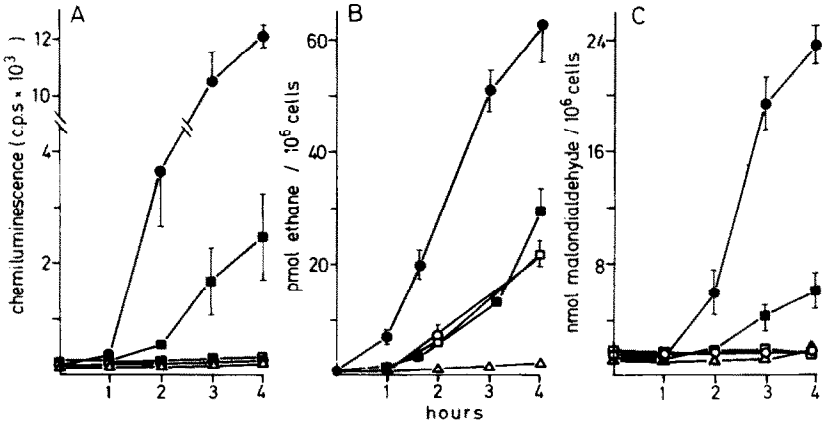

Fig. 3. Effect of different foreign compounds on the rate of lipid peroxidation in isolated hepatocytes. Hepatocytes (10^6 cells/ml) were incubated at 37° in the absence (○) and presence of 2 mM ethylmorphine (□), 5 mM aminopyrine (△), 5 mM carbon tetrachloride (●) and 0.6 mM bromobenzene (■). The amount of lipid peroxidation occurring in the hepatocytes was determined at hourly intervals, for up to 4 hr, using assays of (A) chemiluminescence, (B) ethane production and (C) malondialdehyde formation. Values are expressed as the means (± S.E.M.) of 4 separate experiments. Where error bars are not shown, the S.E.M. lies within the symbols used.

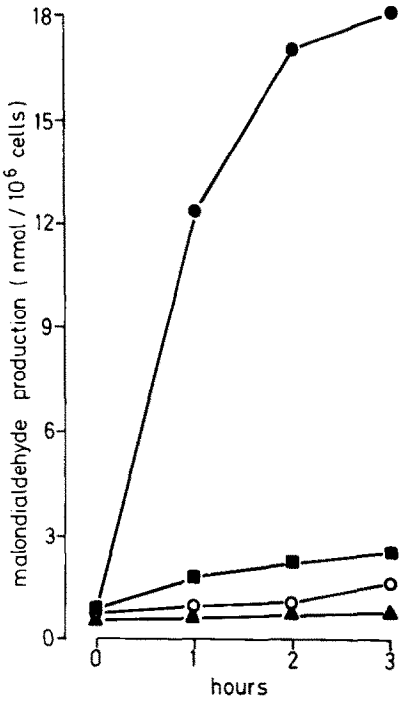

Fig. 4. Effect of u.v. irradiation on the rate of lipid peroxidation in isolated hepatocytes incubated in the absence, (●), and presence of either 2 mM ethylmorphine, (■), or 2 mM aminopyrine, (▲). The amount of lipid peroxidation occurring in control, unirradiated cells is also shown, (○). Hepatocytes (10^6 cells/ml) were irradiated for 5 min in open Petri dishes in the absence or presence of the drug substrate and then incubated in rotating round-bottom flasks as described. Malondialdehyde production was used as the index of lipid peroxidation. The results of one experiment, typical of three, are shown.

of 5 mM aminopyrine tended to inhibit lipid peroxidation rather than stimulate it (Fig. 3). Thus, although drugs, such as ethylmorphine and aminopyrine stimulate H_2O_2 production in isolated hepatocytes [29–32], this does not necessarily lead to an increased rate of lipid peroxidation in the cells. This is probably due to the fact that there are very efficient H_2O_2-metabolizing systems in liver cells [32] and the finding that aminopyrine and ethylmorphine are quite powerful antioxidants [33]. Aminopyrine has been shown to be a more powerful inhibitor of hepatic microsomal lipid peroxidation than ethylmorphine [33] and this is confirmed for whole liver cells by the results shown in Fig. 4. Exposure of hepatocytes to a non-chemical peroxidizing agent such as u.v. light caused a large increase in the rate of lipid peroxidation in the cells during subsequent incubation (Fig. 4). This increase was completely inhibited in the presence of 2 mM aminopyrine but was incompletely inhibited by 2 mM ethylmorphine (Fig. 4). Thus, aminopyrine is a better antioxidant and inhibitor of lipid peroxidation than ethylmorphine.

Relationship between lipid peroxidation and the cytotoxic effects of bromobenzene and carbon tetrachloride

Both carbon tetrachloride and bromobenzene are widely-studied hepatotoxic agents which produce centrilobular hepatic necrosis when administered in sufficient doses to experimental animals [1, 22]. At the present time there is considerable controversy as to the importance of lipid peroxidation in the toxic effects of these compounds [1–3, 22, 30]. From Fig. 3 it can be seen that not only does carbon tetrachloride (5 mM) produce significant amounts of lipid peroxidation in isolated hepatocytes, but that bromobenzene (0.6 mM) also does this after 3–4 hr of incubation. In order to further investigate the possible importance of lipid peroxidation in bromobenzene and carbon tetrachloride hepatotoxicity we decided to investigate the relationship between the time of onset of lipid peroxidation and cell viability in hepatocytes exposed to bromobenzene or carbon tetrachloride. Hepatocytes (10^6 cells/ml) were incubated at 37° in the presence of either carbon tetrachloride (5 mM) or bromobenzene (0.6 mM). The amount of chemiluminescence and ethane produced was measured at 20 min intervals up to 2 hr and then at hourly intervals. Cell viability was assayed at exactly the same times using the NADH penetration assay [24], which monitors the integrity of the hepatocytes plasma membrane and has been shown to be a sensitive index of cell death [23, 24]. The results, summarized in Fig. 5, show that lipid peroxidation occurs at an early stage and prior to cell death in carbon tetrachloride cytotoxicity, but at a much later stage, and probably only after cell death, in bromobenzene cytotoxicity. Although in Fig. 5(B) it appears that the level of ethane production, and thus lipid peroxidation, may be becoming significant after 4 hr incubation with bromobenzene, it should be noted from Fig. 3(B) that this level is only slightly above that seen in the control hepatocytes after 4 hr incubation. The viability of the control hepatocytes, however, is still more than 80 per cent, as determined by the NADH penetration assay (data not shown, cf. Refs 22–24). These results indicate that the lipid peroxidation observed during bromobenzene hepatotoxicity is of secondary importance and possibly occurs only as a consequence of earlier toxic effects of bromobenzene, such as glutathione depletion [22, 34].

DISCUSSION

In recent years suspensions of freshly isolated hepatocytes have proved to be an extremely useful model for studies on drug metabolism and toxicity [22–24]. However, their use in studies or the role of lipid peroxidation in drug toxicity is limited by the lack of sensitive methods for the measurement of lipid peroxidation in these cell suspensions. In the present study different methods for the measurement of lipid peroxidation in isolated hepatocytes have been directly compared and evaluated. Five different methods were used to measure the amount of lipid peroxidation in isolated hepatocytes exposed to carbon tetrachloride and the results obtained by the four most recently developed techniques, namely the assays of fluorescent products, chemiluminescence, ethane and pentane production were directly correlated with those obtained by the commonly-used thiobarbituric acid assay of malondialdehyde for-

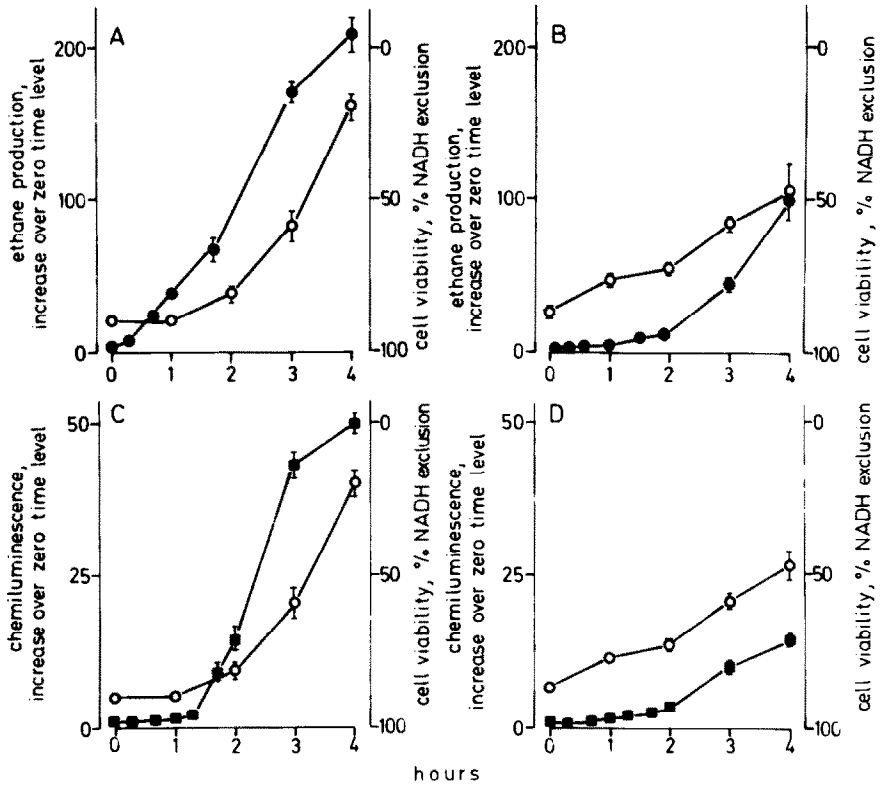

Fig. 5. Relationship between lipid peroxidation and cell toxicity due to bromobenzene and carbon tetrachloride. Hepatocytes (10^6 cells/ml) were incubated at 37° in the presence of either 5 mM carbon tetrachloride (A, C) or 0.6 mM bromobenzene (B, D). The increase in ethane production (●) and chemiluminescence (■) over the zero time level was measured at the same time as cell viability (○) over a 4-hr period. Values represent the means (±S.E.M.) of 4 separate experiments. Where error bars are not shown, the S.E.M. lies within the symbols used.

mation. Measurements of ethane production, chemiluminescence and fluorescent products correlated extremely well with those of malondialdehyde formation under the conditions used in this study (Fig. 1). Previous workers have also found that ethane production correlates closely with carbon-tetrachloride induced malondialdehyde formation in liver homogenates [17], as well as with the production of conjugated dienes *in vivo* [21, 35]. Chemiluminescence initiated in hepatic microsomes by either NADPH or ascorbate is also directly proportional to malondialdehyde formation [18] and Tappel and co-workers [27, 36] have shown that fluorescent product formation is directly proportional to the production of thiobarbituric acid reactive carbonyls in liver mitochondria and microsomes following peroxidative damage. These findings therefore lead us to conclude that measurements of ethane production, chemiluminescence and fluorescent products are valid indices of lipid peroxidation in isolated hepatocytes.

Earlier studies [27] in chemical systems showed a close correlation between peroxide values and pentane released after thermal decomposition of soybean oil hydroperoxides, but in the present study only a relatively poor correlation (r=0.850) was found between pentane production and malondialdehyde formation in isolated hepatocytes. This poor correlation probably reflects varying levels of further metabolism of both n-pentane and malondialdehyde in isolated hepatocytes since rodents are known to metabolize n-pentane at a relatively high rate [15] and n-pentane has also been shown to disappear from a sealed chamber when an isolated perfused rat liver is present [16]. However, our results may also reflect variations in the amount of n-pentane released into the head space above the cell incubate, because pentane is slightly soluble in aqueous solutions and only half of the pentane produced by the peroxidizing cells was released into the head space at 37° under the conditions we have described. In contrast to pentane, ethane is totally insoluble in aqueous solutions, thus it is released and accumulates in the head space above the cell incubate, and is not further metabolized by liver cells [15, 16]. Ethane production is therefore a far more reliable index of lipid peroxidation in isolated liver cells as compared with pentane production.

The sensitivity of the five different assays has also been tested in the present study by expressing the values obtained at specific time intervals as the increase over the zero time level. Ethane production was by far the most sensitive index of lipid peroxidation in isolated hepatocytes and the sensitivity of the chemiluminescence assay was also good. The use of chemiluminescence as an index of drug-induced lipid peroxidation in isolated hepatocytes is, however, limited by the fact that the measurements can

only be expressed in arbitrary units and there is also the potential problem of quenching by the drug under investigation. Ethane production, on the other hand, can be expressed in absolute terms and it may therefore be possible to calculate the extent of fatty acid destruction in the peroxidizing liver cells, which has been done with absolute measurements of malondialdehyde formation in isolated microsomes [38, 39]. A limitation of ethane measurement as an index of lipid peroxidation in isolated cells is, however, that the cells have to be incubated in sealed flasks under initially unphysiological hyperoxic conditions, in order to prevent anoxia during subsequent incubation. The problem of oxygen supply also means that relatively short incubation times have to be used. This tends to restrict the use of a more physiological complete incubation medium, instead of the balanced buffered salts medium, used in the experiments described here, because the use of such a medium tends to delay toxic effects, which are seen at earlier times in the incomplete balanced salts medium [22, 24]. We are presently trying to overcome the problem of inadequate oxygen supply in this experimental system during long term incubation.

In comparison to ethane measurement, the assays of malondialdehyde and fluorescent product formation, and of pentane production, were relatively insensitive and Figs. 2 and 3 show that no significant increase over the zero time level was found in any of these three parameters until after 90 min of incubation in the presence of carbon tetrachloride, whereas a significant increase in ethane production over the zero time level was observed after only 20–40 min incubation. This insensitivity is probably due to the further metabolism of malondialdehyde [11, 12] and pentane [15, 16] in intact liver cells. Moreover, the fluorescent products (conjugated Schiff bases) formed during lipid peroxidation are believed to derive from reactions between malondialdehyde and amino acids or their esters [13, 14].

Measurements of ethane production and chemiluminescence are therefore the most useful techniques for measuring low levels of lipid peroxidation in isolated hepatocytes. We therefore decided to use these two methods, along with the more established thiobarbituric acid assay, to determine whether or not the drug-stimulated production of H_2O_2 leads to an increased rate of lipid peroxidation in isolated liver cells. It is well-established that various drugs, such as aminopyrine and ethylmorphine, stimulate H_2O_2 production in isolated hepatocytes and that there is a concomitant loss of cellular glutathione [29–32]. One of the implications of this oxidative stress may be an increase in the rate of lipid peroxidation within the liver cells, but in the present study no increase in ethane, chemiluminescence or malondialdehyde production was observed during incubation of isolated hepatocytes with ethylmorphine and aminopyrine at concentrations known to stimulate H_2O_2 production. However, the results of several studies [40, 41] have shown that both aminopyrine and ethylmorphine are potent inhibitors of lipid peroxidation in liver microsomes, and recent studies [33] have shown that this is directly due to their antioxidant properties (see also Fig. 4). The antioxidant properties of these drugs may therefore have prevented any lipid peroxidation resulting from H_2O_2 accumulation. Other drugs, which stimulate H_2O_2 production by the cytochrome P-450 system, but lack significant antioxidant properties, may therefore cause toxicity by stimulated formation of reactive oxygen species, including H_2O_2. However, liver cells have many systems to protect them from reactive oxygen species, including superoxide dismutase, natural antioxidants and very efficient H_2O_2-metabolizing enzyme systems [30, 31]. The formation of reactive oxygen species as a mechanism of drug toxicity therefore remains highly controversial [4–8, 30].

Finally, the results of experiments described in this study further indicate that the onset of lipid peroxidation is an important event in carbon tetrachloride hepatotoxicity, but that it appears to be only a subsequent event in bromobenzene cytotoxicity, possibly only occurring as a result of cell necrosis. These findings are in agreement with those of previous workers [42, 43] and show that the use of sensitive indices of lipid peroxidation in isolated cells, such as ethane production and chemiluminescence, can yield valuable information regarding the primary toxic effects of foreign compounds. These methods are presently being applied in our laboratory in studies on the toxic effects of xenobiotics, such as paraquat and adriamycin, where the role of lipid peroxidation remains unclear.

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