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Interaction of Hypoxia and Carbon Tetrachloride Toxicity in Hepatocyte Monolayers

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The toxicity of carbon tetrachloride (CCl_4) in monolayer cultures of primary hepatocytes was investigated at oxygen concentrations that prevail in the liver under conditions that range from normoxia to hypoxia: 0.5, 1, 2, and 20% O_2. CCl_4 was administered in the vapor phase at concentrations that produce aqueous concentrations at 37°C of 0.4, 2.0, and 4.0 m*M*. Damage was assayed by leakage of aspartate transaminase and the inclusion of Trypan Blue immediately after the 2-hr incubation and after an additional 6-hr incubation in 20% O_2. Only in the case of 0.5% O_2 and 4 m*M* CCl_4 were the monolayers damaged (18%) immediately after the 2-hr exposure; all other exposed cells were undamaged at that time point and the dose response of cell death as a function of CCl_4 and oxygen concentration was not evident until the 6-hr time point. The monolayers exposed to 4 m*M* CCl_4 and 1, 2, or 20% O_2 exhibited little immediate damage but were all 100% dead 6 hr later. The monolayers exposed to 2 m*M* CCl_4 and 0.5, 1, 2, or 20% O_2 were 53, 48, 40, and 22 ± 2% dead after 6 hr, respectively. These results suggest that effects of CCl_4 exposure, for example alterations in the function or synthesis of essential proteins, require several hours to affect cell viability.

INTRODUCTION

Carbon tetrachloride (CCl_4), long known to cause hepatic centrilobular necrosis (Cameron and Karunaratne, 1936), is regarded as a classic hepatotoxin; its toxicity requires hepatic metabolism and involves free radical formation (Butler, 1961), lipid peroxidation (Rao and Recknagel, 1968), phosgene formation (Kubic and Anders, 1980), and disturbances in Ca^{2+} homeostasis (Moore, 1980; Long and Moore, 1987). However, the actual causal events leading to cell death have not been defined (Berger *et al.*, 1987; Mico *et al.*, 1982). Two findings implicate the prevailing oxygen concentration as an important factor in CCl_4 toxicity: *In vivo* CCl_4 toxicity is exacerbated by hypoxia, and necrosis is consistently greatest around the central vein of the liver. It has been established that the centrilobular areas of the liver lobule seldom experience oxygen concentrations above 4% (40 μ*M*) under conditions of normal blood flow (Baraona *et al.*, 1983). Any conditions that reduce hepatic blood flow or decrease the oxygen saturation of blood result in far lower oxygen concentrations in this area of the liver lobule. It has also been shown that hypoxia alone can cause liver cell death (Shingu *et al.*, 1982). In fact, it has been suggested that some halocarbons, such as halothane, are hepatotoxic because they reduce liver blood flow or increase oxygen utilization rather than because of their metabolism (Jones, 1981; Shingu *et al.*, 1982).

On the other hand, under low oxygen concentrations cytochrome P450 switches from oxidative metabolism of CCl_4, where chloroform, phosgene, and carbon dioxide (Kubic and Anders, 1980; LaCagnin *et al.*, 1988) are major metabolites, to reductive metabolism where the trichloromethyl free radical is a major product (Butler, 1961; Noguchi *et al.*, 1982). As early as 1961 Butler predicted that a free

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radical could be formed *in vivo* from CCl_4 (Butler, 1961). The existence of the trichloromethyl free radical has since been demonstrated by ESR spin-trapping techniques (Noguchi *et al.*, 1982). In addition, we have performed reductive metabolism of CCl_4 with cytochrome P450 and NADPH–cytochrome P450 reductase reconstituted into phospholipid vesicles containing dioleoylphosphatidylcholine and isolated an adduct of the trichloromethyl radical with an oleoyl fatty acid chain which was characterized by mass spectrometry (Trudell *et al.*, 1982). The trichloromethyl free radicals can abstract hydrogen radicals from fatty acid chains of cellular membranes. The resulting carbon-centered radicals on the fatty acids react rapidly with oxygen to form peroxy radicals that initiate a chain reaction of lipid peroxidation (Recknagel, 1983). The oxygen dependence of this peroxidation has been studied; the maximum rate occurs below 1% O_2 in isolated hepatocytes (de Groot and Noll, 1986). Clearly, the binding of free radicals to essential intracellular components as well as the detrimental effect of lipid peroxidation and the subsequent release of oxidized lipids must be included in a model of CCl_4 toxicity.

It is difficult to study the effects of hypoxia on CCl_4 metabolism *in vivo* or in a perfused liver preparation because of the direct effects of CCl_4 on hepatic blood flow and because of the steep oxygen gradient across the liver lobule. This gradient is typically from 100 μM in the periportal area to 40 μM at the pericentral vein; under conditions of severe hypoxia or reduced blood flow, the concentration around the central vein can be less than 1 μM (Ji *et al.*, 1983; Baraona *et al.*, 1983). Therefore, it is not possible in an intact liver to expose all hepatocytes to the same oxygen concentration; the results of metabolic damage will be an average over cells exposed to a range of concentrations. The role of hypoxia in CCl_4 toxicity can be determined using primary cultures of hepatocyte monolayers exposed in the well-defined oxygen environments. While there is general consensus on the potentiation of CCl_4 toxicity under low oxygen tensions, a report has also appeared where there was no increase in CCl_4 toxicity under hypoxic conditions (Suarez *et al.*, 1972).

In order to establish the importance of hypoxia in the toxicity of CCl_4 and to measure the effect of moderate damage that may require hours to manifest, monolayer cultures of primary hepatocytes were exposed to oxygen concentrations that range from 0.5, 1, 2, to 20% and to CCl_4 in the vapor phase at 12,000, 60,000, and 120,000 ppm that produced aqueous concentrations at 37°C of 0.4, 2.0, and 4.0 mM, respectively. Damage was assessed immediately after the exposure as well as 6 hr later. It was found that hypoxia potentiated the toxicity of CCl_4 that became evident only after an additional 6-hr incubation at 20% O_2.

METHODS

Hepatocyte Isolation

Hepatocytes were prepared from male Fisher 344 rats (180–240 g) by *in situ* perfusion of the liver with collagenase as previously described (Bissell and Guzelian, 1980; Schieble *et al.*, 1988). The isolated and washed hepatocytes were suspended in 100 ml of a medium (complete M-199) containing GIBCO M-199 with Earle's salts, pH 7.3, 100 U penicillin/ml, 100 μg streptomycin/ml, 4.5 μg gentamicin/ml, 1 μg transferrin/ml, 0.25 mM ascorbic acid, 4.12 μM folic acid, 1.24 mM pyruvate, 8 U insulin/ml, 100 μM 5-aminolevulinic acid, 0.25% bovine serum albumin, and 10% heat-inactivated fetal calf serum (GIBCO). The 5-

aminolevulinic acid has been shown to maintain total cytochrome P450 content at *in vivo* levels (Paine and Hockin, 1980). One-milliliter aliquots containing approximately 3×10^6 cells were added to collagen-coated 60-mm Lux Permanox Contur (Flow Laboratories) culture dishes and allowed to attach for 120 min at 37°C in 5% CO_2/95% air. These culture dishes were preincubated at 37°C for 3 hr in complete M-199 with 20% fetal calf serum and 1 μM dexamethasone to form a matrix, which facilitates attachment, prior to addition of cell suspension aliquots. The high gas permeability of the Permanox dishes permits rapid equilibration of the cells and medium with any desired gaseous environment. The hepatocyte monolayers were then washed twice after the 120-min attachment period and covered with 2 ml complete M-199. Cells at this point were more than 95% viable as assayed 5–15 min after addition of Trypan Blue by observing five fields of approximately 200 cells at 200× magnification with a Nikon Diaphot inverted microscope. Cytochrome P450 content at isolation and 24 hr after plating was ascertained as described previously (Schieble *et al.*, 1988). Previous studies in this laboratory, involving identically prepared hepatocytes, demonstrated that the metabolic activity of these cells was unchanged after 3.5 hr of 2% O_2 hypoxia (Gut *et al.*, 1986).

Experimental Protocol

The CCl_4 exposure experiments were initiated 18 hr following isolation and plating of the hepatocytes. Prior to each CCl_4 or control exposure, the monolayers were washed twice with buffer and covered with 2 ml M-199 medium without fetal calf serum and bovine serum albumin and then checked for confluency with an inverted microscope. Cells were then removed from a standard incubator (5% CO_2/95% air at 37°C) and placed in a Billups–Rothenberg modular incubator chamber for exposure to CCl_4 and/or selected hypoxic gas mixtures. The incubator chamber was altered to permit the gas mixture to flow in from the top of the chamber onto the surface of a 100-mm petri dish filled with sterile H_2O that was placed on the top shelf. The resulting turbulence caused a uniform gas flow over five culture dishes placed on each of the two lower shelves. The outflow port at the incubator chamber was extended upward to the surface with tubing, and the entire sealed incubator chamber was immersed in a 37°C water bath.

In order to expose the cells to stable concentrations of CCl_4 over the 2-hr exposure period at the various oxygen tensions, the CCl_4 was administered in the vapor phase. The concentrations in the vapor phase and the medium were determined by gas chromatography with a Varian 2100 gas chromatograph on a 30 m × 0.053 mm (i.d.) DX-624 column (J & W Scientific) with a helium flow of 10 ml/min. Column, injector, and detector temperatures were 20, 50, and 50°C, respectively. Standards of CCl_4 were made up in carbon disulfide to prepare a calibration curve. The CCl_4-saturated vapor from the copper kettle vaporizer was bubbled into complete M-199 at 20 and 37°C for 30 min. Similar solutions were equilibrated with mixtures of CCl_4-saturated vapor mixed 1:1 and 1:9 with 20% O_2/5% CO_2/balance N_2. Aliquots (250 μl) of the supernatants of duplicate hepatocyte monolayers exposed to CCl_4 were withdrawn through ports drilled in the top of the exposure chamber. They were immediately mixed 1:1 with carbon disulfide, the layers were allowed to separate, and 1 μl of the carbon disulfide layer was injected into the gas chromatograph. The efficiency of this extraction was found to be 65%.

The gas mixtures of 0.5, 1, 2, or 20% O_2/5% CO_2/balance N_2 were made with

mass flow controllers (Porter Instrument Co., Inc.) at 0.75 liter/min; a metering valve was used to direct a percentage of the flow through a copper kettle vaporizer to form the desired concentrations of CCl_4. An oxygen monitor (OHMEDA 5100, B.O.C. Health Care), calibrated against room air and various O_2/N_2 mixtures, was used to measure the desired O_2 concentration. The highest concentration of CCl_4 (4 m*M*) was the result of the entire flow going through the vaporizer at 20°C. The gas mixture or gas/CCl_4 mixture was passed through a glass frit humidifier that humidified and warmed it to 37°C before entry into the exposure chamber. All groups of dishes remained in the incubator chamber for a 2-hr exposure with lids off, and experiments in the different groups were performed sequentially throughout the day, with the controls done before the chamber was exposed to CCl_4. Immediately following the experimental exposure, supernatants were removed from 5 of the 10 identical monolayers in each group. The supernatants were centrifuged to separate dead cells and debris, and the resulting supernatants were analyzed for aspartate aminotransferase (AST) release using Sigma Kit No. 505. A supplementary assessment of cell death was obtained by measuring Trypan Blue exclusion of the cells following removal of the supernatant. The remaining five dishes were incubated in 5% CO_2/95% air at 37°C for 6 hr. At the end of this 6-hr period, the supernatants from these dishes were tested for AST release and Trypan Blue exclusion as described above.

The final aqueous concentration of complete M-199 equilibrated at 25 or 37°C with the gas mixture saturated with CCl_4 at 20°C, as measured by gas chromatography, was 5.0 ± 0.5 or 4.0 ± 0.5 m*M*, respectively. These values compare well with the 5.2 m*M* at 20°C previously reported (Mackison *et al.*, 1981) and with the approximately 1 m*M* decrease in solubility at 37°C that would be predicted for a molecule with an enthalpy of transfer into water of −2000 cal/mole (Tanford, 1973). In order to test the evaporation rate of CCl_4 from plates, 2 ml of either 4 or 2 m*M* CCl_4 in complete M-199 were added to 60-mm Lux Permanox plastic tissue cultures dishes; they were allowed to stand uncovered in a Forma incubator at 37°C for 5 min, and duplicate determinations of CCl_4 concentration were made. When it was found that 81 ± 2% of the CCl_4 had evaporated, the determination was repeated in 60-mm Pyrex petri dishes with identical results. The time required to reach equilibrium with CCl_4-saturated vapor was determined by exposing Lux Permanox dishes containing 2 ml of complete M-199 in the incubator for 15, 30, 60, and 120 min. It was found that complete equilibrium was obtained within 15 min and that the values at later times were not significantly different by an *F* test. A separate trial experiment demonstrated that equilibrium with 1 and 2% O_2 concentrations in the 2 ml of culture media in the Permanox dishes was obtained at 20 and 8 min, respectively.

Data Analysis

Experiments were performed on two separate hepatocyte preparations (total $n = 10$ for each point) to average dish-to-dish and rat-to-rat variations. The AST release for each dish was determined as described in the Sigma Kit No. 505, compared to complete AST release caused by lysis with 0.2% Triton X-100, and expressed as a percentage. It should be noted that 80% cell lysis, as measured by AST release, corresponded to 100% cell death as monitored by the cells' inability to exclude Trypan Blue. Estimates of the percentage of cells taking up Trypan Blue were used for comparison with AST measurements of cell death, but were

not entered into the data analysis directly because of their subjectivity. In previous studies we have shown an excellent correlation between ^{51}Cr leakage and AST release as a measure of cell death (Costa *et al.*, 1987a). Multiple regression was chosen as the method of data analysis because of the study design which included three independent variables (oxygen concentration, CCl_4 concentration, and time after exposure) and a single dependent variable (% cell death). In order to have a correlation of oxygen concentration versus cell death with a positive sign, oxygen concentration was transformed to hypoxia = 20% O_2/experimental % O_2. The data were analyzed using Statworks software (Cricket Software) on an Apple MacIntosh II computer.

RESULTS

Figure 1 shows a completely confluent hepatocyte monolayer 24 hr following isolation and plating. We have found that nearly completely confluent monolayers are essential for reproducible results in these studies. Cells attached individually or in small patches have an unpredictable response to hypoxia. Figure 2 shows an identical monolayer following a 2-hr exposure to 4 m*M* CCl_4 and 20% O_2. There are large gaps and refractile areas in the CCl_4-exposed hepatocytes compared to those in the control monolayer. Although these exposed hepatocytes still excluded Trypan Blue and did not release appreciable levels of AST (Fig. 3a), an irreversible event had occurred that resulted in 100% cell death 6 hr later (Fig. 3b). Very little damage was apparent immediately following the 2-hr exposure of the hepatocyte monolayers to both hypoxia and CCl_4, as gauged from their ability to exclude Trypan Blue and the lack of release of AST (Fig. 3a). Only at 4 m*M* CCl_4

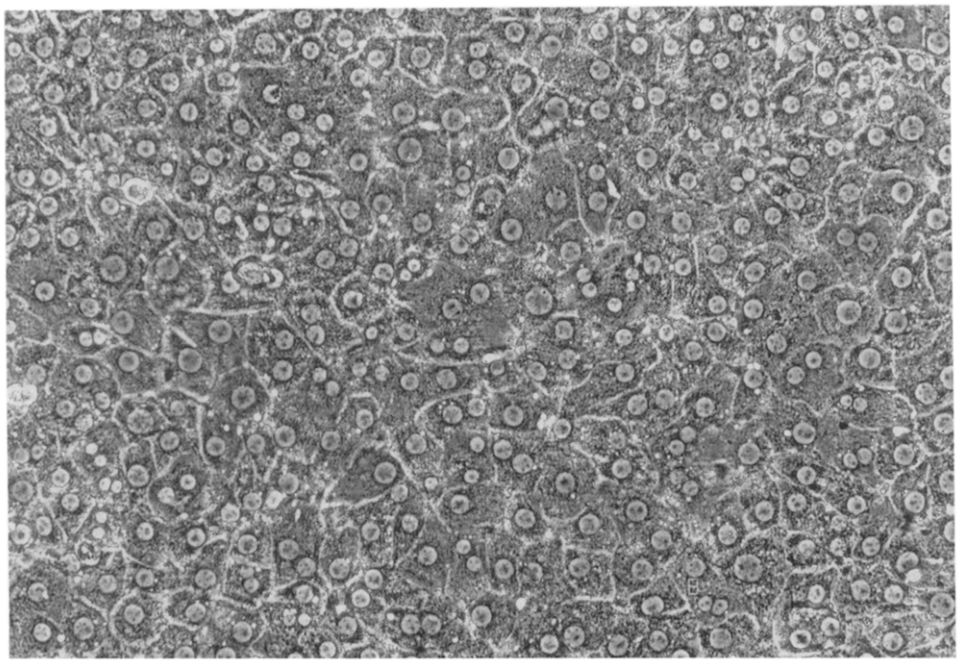

FIG. 1. Healthy confluent monolayer of rat hepatocytes with good cell-to-cell contact 26 hr after isolation and plating (phase contrast, magnification 175×).

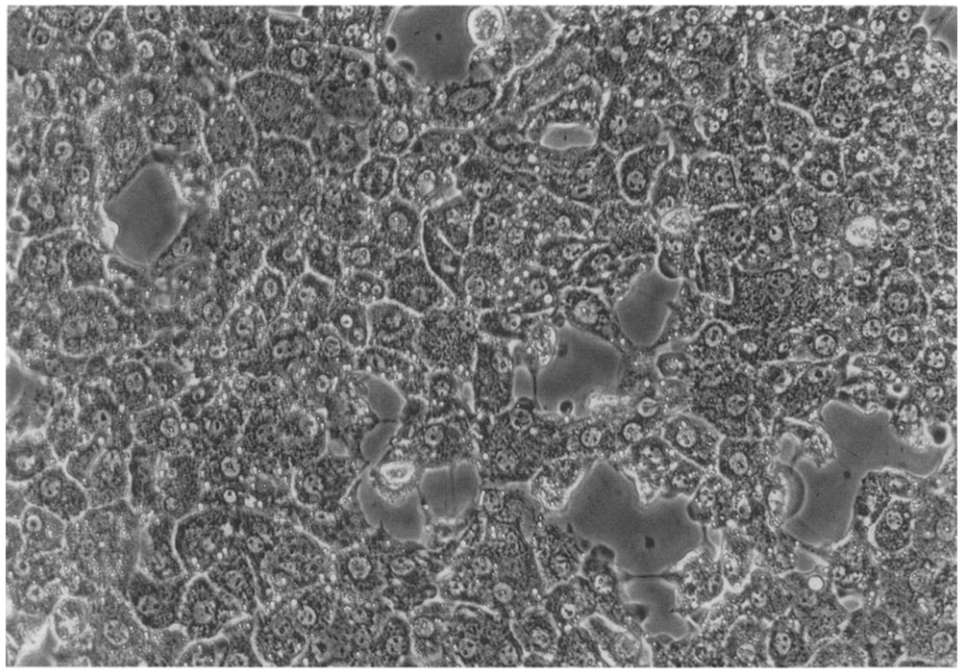

FIG. 2. Typical view of a hepatocyte monolayer following exposure to 5 m*M* CCl_4 and 20% O_2 for 2 hr. The cells are rounding up, blebs appear as spots in the cytosolic area, and there are definite gaps where cells have lifted (phase contrast, magnification 175×).

and 0.5% O_2 was there evidence of slightly more damage following exposure than their normoxic counterparts (Fig. 3b).

However, after a further 6 hr, extensive cell damage was observed and all the monolayers exposed to 4 m*M* CCl_4 were 100% dead irrespective of the oxygen concentration they experienced during the CCl_4 exposure (Fig. 3b). The monolayers exposed to 2.0 m*M* CCl_4 all exhibited damage at 6 hr following the exposure; the monolayers exposed to 0.5% O_2 were significantly more damaged ($P < 0.05$) than those exposed to 2% O_2. The latter, in turn, were significantly more damaged than those exposed to 2.0 m*M* CCl_4 and 20% O_2 ($P < 0.01$). At the lowest exposure of CCl_4 (0.4 m*M*) and 0.5% O_2, the monolayers were 30% lysed after 6 hr, while monolayers treated with 0.4 m*M* CCl_4 and 1, 2, or 20% O_2 showed no signs of damage at 6 hr (Fig. 3b). The results reported here regarding AST release and Trypan Blue exclusion at 6 hr following CCl_4 exposure at all three concentrations were similar to studies that were carried out under identical conditions but the monolayers were incubated for 24 hr following exposure (data not shown), indicating that the damage was fully manifest by the 6-hr time point.

Analysis of the data by multiple regression quantified the effects presented in Fig. 3. The data points were divided into 0 and 6-hr groups (120 points in each group) and then each group was analyzed by multiple regression with hypoxia and CCl_4 concentration as independent variables. At 0 hr there was no significant effect (the coefficient of determination [R^2] was only 0.354). At 6 hr both hypoxia (coefficient 0.413) and CCl_4 (coefficient 15.995) made significant contributions to toxicity and the coefficient of determination (R^2) for the effect of the two variables was 0.854.

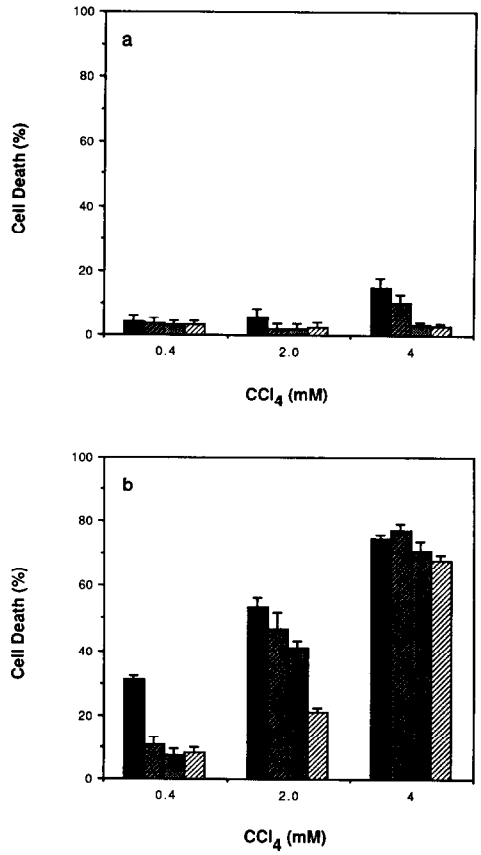

FIG. 3. (a) Percentage cell death ± SE in monolayers of hepatocytes immediately following a 2-hr exposure to 0.5% (■), 1% (▨), 2% (▩), and 20% (▨) oxygen in the presence of varying amounts of CCl_4. Percentage cell death was obtained by dividing leakage of AST under the experimental conditions by the leakage of AST in the presence of 0.02% Triton X-100 times 100. In each case the AST leakage in monolayers exposed to the corresponding oxygen concentration alone was subtracted. (b) Percentage cell death (±SE) in monolayers of hepatocytes incubated an additional 6 hr at 20% O_2 following a 2-hr exposure to 0.5% (■), 1% (▨), 2% (▩), 20% (▨) oxygen in the presence of varying amounts of CCl_4. In each case the AST leakage in monolayers exposed to the corresponding oxygen concentration alone was subtracted.

Interesting qualitative observations were as follows: Results with Trypan Blue exclusion by the hepatocyte monolayers generally were in agreement with AST release and reflected the status of the monolayers as observed with phase contrast microscopy, except in the case of the highest CCl_4 concentration immediately following exposure. The latter monolayers were more damaged as assessed by Trypan Blue exclusion and by phase microscopy than as measured by AST release. Hepatocytes were pulling apart from neighboring cells, they were more refractile, and the nuclei lost their round appearance. However, it was found that virtually no lysis of the monolayers had occurred as monitored by the release of AST. Only slight damage was evident by AST release immediately after exposure to 0.5% O_2 and 4 m*M* CCl_4 (18%) and, accordingly, these monolayers appeared more damaged by phase contrast microscopy and by Trypan Blue exclusion than the monolayers exposed to equal concentrations of CCl_4 and higher oxygen con-

centrations. Furthermore, not evident either by the Trypan Blue exclusion assay or by the AST release was the physical state of the monolayers following exposure to 4 m*M* CCl_4; as the oxygen concentration and the viability increased, so did the amount and size of gaps in the monolayer (Fig. 2), indicating individual cell lifting. The absence of AST release is in agreement with work of Chenery *et al.* (Chenery *et al.*, 1981) where cell lifting occurred without corresponding enzyme release. At the 6-hr time point, although all monolayers failed to exclude Trypan Blue and released most of their AST, significant differences were apparent with phase contrast microscopy. Monolayers exposed to 4 m*M* CCl_4 and 0.5% O_2, in which no gaps were seen immediately after the exposure, lifted up from the dish as completely intact dead monolayers. On the other hand, monolayers exposed to CCl_4 and higher oxygen concentrations (1.0, 2.0, and 20%), in which some gaps were evident immediately after exposure, lifted off as small segments after 6 hr.

DISCUSSION

It has been shown that in standard media the levels of cytochrome P450 drop considerably after 24 hr in culture (Bissell and Guzelian, 1980). However, Paine and Hockin (Paine and Hockin, 1980) found that by the addition of 5-aminolevulinic acid the total level of the cytochrome P450 could be maintained in cultured hepatocyte monolayers. We routinely add 100 μ*M* 5-aminolevulinic acid, as well as insulin and dexamethasone, to our medium and have found that the cytochrome P450 levels 24 hr after isolation are maintained at the same levels as found *in vivo* (Schieble *et al.*, 1988).

In that equilibrium of the complete M-199 with CCl_4-saturated vapor was obtained in 15 min in a Lux dish placed in the incubator, it is clear that exposure to CCl_4 vapor is an effective method of obtaining a stable CCl_4 concentration (Tyson *et al.*, 1983). In contrast, the result that 81 ± 5% of the CCl_4 was lost in 5 min from complete M-199 in either a Lux Permanox or a Pyrex culture dish suggests that injection of a single dose of CCl_4 into the medium results in a rapidly changing and quickly depleted concentration when used in a system that is not gastight. This is an important point to consider in comparing the results of the present study with those of other studies in which CCl_4 was delivered as a single dose.

The most notable feature of this study was that cell damage was not obvious immediately following the 2-hr exposure. In fact, at this time point the CCl_4-exposed hepatocyte monolayers appeared nearly identical to similarly treated control monolayers by two indices, AST release and Trypan Blue exclusion. The only sets of monolayers that showed slight damage immediately following the exposure were at the highest CCl_4 concentrations (4m*M*) and the lowest O_2 concentration (Fig. 3a). However, at the 6-hr time point, extensive cell death was demonstrated by both of the indices above (Fig. 3b).

These results are somewhat different from those of Tyson *et al.* who reported that the ED_{50} of CCl_4 was 2 m*M* in a suspension of primary hepatocytes immediately after a 2-hr exposure to a constant vapor phase of CCl_4 (Tyson *et al.*, 1983). Chenery *et al.* (Chenery *et al.*, 1981) reported an ED_{50} of between 2 and 3.6 m*M* CCl_4 following a 2-hr incubation with freshly cultured hepatocytes (1 hr old at initiation of the experiment). It is likely that these differences in time course are due to the increasing stability of hepatocytes in monolayer culture versus in suspension. Following 12–24 hr in monolayer culture, hepatocytes exhibit gap junctions, desmosomes, normal protein synthesis, and cytosolic calcium levels

return to the *in vivo* range of 100 n*M* (Lemasters *et al.*, 1987). The longer life span of hepatocyte monolayers versus hepatocyte suspensions was of considerable advantage in the present study as the acute toxicity was not discernable immediately and the dose–response curve of CCl_4 toxicity as a function of the oxygen concentration was only evident at 6 hr following exposure. This time dependence observed in tissue culture is consistent with the time course of *in vivo* histopathology following exposure to CCl_4 (Cameron and Karunaratne, 1936) or halothane (Ross *et al.*, 1979).

In these hepatocyte monolayers, CCl_4 was found to be more toxic at 0.5% O_2 than at 20% O_2 for all concentrations of CCl_4 studied. Analysis of the 240 data points by multiple regression showed that at 6 hr after exposure, CCl_4 concentration and hypoxia were statistically significant factors contributing to cell death. It has been shown that lipid peroxidation caused by CCl_4 in isolated hepatocytes is maximum at approximately 0.65% O_2 (de Groot and Noll, 1986), that no peroxidation occurs under anoxic conditions, and that the rate is reduced precipitously above 1.3% O_2. Thus, the results of this study are consistent with formation of free radicals or peroxidation of lipids being major factors in the increased toxicity of CCl_4 at low oxygen concentrations. They are also in accord with our previous studies with the calcium ionophore A23187 (Costa *et al.*, 1987a) and *t*-butyl hydroperoxide (Costa *et al.*, 1987b) in which it was concluded that hypoxia predisposed hepatocytes to damage.

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REFERENCES

BARAONA, E., JAUHONEN, P., MIYAKAWA, H., and LIEBER, C. S. (1983). Zonal redox changes as a cause of selective perivenular hepatotoxicity of alcohol. *Pharmacol. Biochem. Behav.* **18**, 449–454.

BERGER, M. L., REYNOLDS, R. C., and COMBES, B. (1987). Carbon tetrachloride-induced morphologic alterations in isolated rat hepatocytes. *Exp. Mol. Pathol.* **46**, 245–257.

BISSELL, D. M., and GUZELIAN, P. S. (1980). Phenotypic stability of adult rat hepatocytes in primary monolayer culture. *Ann. N.Y. Acad. Sci.* **341**, 85–98.

BUTLER, T. C. (1961). Reduction of carbon tetrachloride *in vivo* and reduction of carbon tetrachloride and chloroform *in vitro* by tissues and tissue constituents. *J. Pharmacol. Exp. Ther.* **134**, 311–319.

CAMERON, G. R., and KARUNARATNE, W. A. E. (1936). Carbon tetrachloride cirrhosis in relation to liver regeneration. *J. Pathol. Bacteriol.* **42**, 1–21.

CHENERY, R., GEORGE, M., and KRISHNA, G. (1981). The effect of ionophore A23187 and calcium on carbon tetrachloride-induced toxicity in cultured rat hepatocytes. *Toxicol. Appl. Pharmacol.* **60**, 241–252.

COSTA, A. K., SCHIEBLE, T. M., HEFFEL, D. F., and TRUDELL, J. R. (1987a). Toxicity of calcium ionophore A23187 in monolayers of hypoxic hepatocytes. *Toxicol. Appl. Pharmacol.* **87**, 43–47.

COSTA, A. K., HEFFEL, D. F., SCHIEBLE, T. M., and TRUDELL, J. R. (1987b). Toxicity of *t*-butyl hydroperoxide in hepatocyte monolayers exposed to hypoxia and reoxygenation. *In Vitro* **23**, 501–506.

DE GROOT, H., and NOLL, T. (1986). The crucial role of low steady state oxygen partial pressures in haloalkane free radical-mediated lipid peroxidation. *Biochem. Pharmacol.* **35**, 15–19.

GUT, J., COSTA, A. K., and TRUDELL, J. R. (1986). Oxygen concentration-dependent metabolism of leukotriene B_4 by hepatocyte monolayers. *Biochim. Biophys. Acta* **878**, 194–199.

JI, S., LEMASTERS, J. J., CHRISTENSEN, V., and THURMAN, R. G. (1983). Selective increase in pericentral oxygen gradient in perfused rat liver following ethanol treatment. *Pharmacol. Biochem. Behav.* **18**, 439–442.

JONES, D. P. (1981). Hypoxia and drug metabolism. *Biochem. Pharmacol.* **30,** 1019–1023.

KUBIC, V. L., and ANDERS, M. W. (1980). Metabolism of carbon tetrachloride to phosgene. *Life Sci.* **26,** 2151–2155.

LACAGNIN, L. B., CONNOR, H. D., MASON, R. P., and THURMAN, R. G. (1988). The carbon dioxide anion radical adduct in the perfused rat liver: Relationship to halocarbon-induced toxicity. *Mol. Pharmacol.* **33,** 351–357.

LEMASTERS, J. J., DEGUISEPPI, J., NIEMINEN, A.-L., and HERMAN, B. (1987). Blebbing, free Ca^{2+} and mitochondrial membrane potential preceding cell death in hepatocytes. *Nature (London)* **325,** 78–81.

LONG, R. M., and MOORE, L. (1987). Cytosolic calcium after carbon tetrachloride, 1,1-dichloroethylene, and phenylephrine exposure. *Biochem. Pharmacol.* **36,** 1215–1221.

MACKISON, F. W., STRICOFF, R. S., and PARTRIDGE, L. J. (1981). "NIOSH/OSHA—Occupational Health Guidelines for Chemical Hazards," DHHA (NIOSH) Publication No. 81-123. U.S. Government Printing Office, Washington, DC.

MICO, B. A., BRANCHFLOWER, R. V., POHL, L. R., PUDZIANOWSKI, A. T., and LOEW, G. H. (1982). Oxidation of carbon tetrachloride, bromotrichloromethane, and carbon tetrabromide by rat liver microsomes to electrophilic halogens. *Life Sci.* **30,** 131–137.

MOORE, L. (1980). Inhibition of liver-microsome calcium pump by *in vivo* administration of CCl_4, CHCl3 and 1,1-dichloroethylene (vinylidene chloride). *Biochem. Pharmacol.* **29,** 2505–2511.

NOGUCHI, T., FONG, K.-L., LAI, E. K., ALEXANDER, S. S., KING, M. M., OLSON, L., POYER, J. L., and MCCAY, P. B. (1982). Specificity of a phenobarbital-induced cytochrome P-450 for metabolism of carbon tetrachloride to the trichloromethyl radical. *Biochem. Pharmacol.* **31,** 615–624.

PAINE, A. J., and HOCKIN, L. J. (1980). Nutrient imbalance causes the loss of cytochrome P-450 in liver cell culture: Formulation of culture media which maintain cytochrome P-450 at *in vivo* concentrations. *Biochem. Pharmacol.* **29,** 3215–3218.

RAO, K. S., and RECKNAGEL, R. O. (1968). Early onset of lipoperoxidation in rat liver after carbon tetrachloride administration. *Exp. Mol. Pathol.* **9,** 271–278.

RECKNAGEL, R. O. (1983). A new direction in the study of carbon tetrachloride hepatotoxicity. *Life Sci.* **33,** 401–408.

ROSS, W. T., JR., DAGGY, B. P., and CARDELL, R. R., JR. (1979). Hepatic necrosis caused by halothane and hypoxia in phenobarbital-treated rats. *Anesthesiology* **51,** 327–333.

SCHIEBLE, T. M., COSTA, A. K., HEFFEL, D. F., and TRUDELL, J. R. (1988). Comparative toxicity of halothane, isoflurane, hypoxia, and phenobarbital induction in monolayer cultures of rat hepatocytes. *Anesthesiology* **68,** 485–494.

SHINGU, K., EGER, E. I., II, and JOHNSON, B. H. (1982). Hypoxia *per se* can produce hepatic damage without death in rats. *Anesth. Analg.* **61,** 820–823.

SUAREZ, K. A., CARLSON, G. P., and FULLER, G. C. (1972). Effect of carbon monoxide or hypoxia on CCl_4 hepatotoxicity. *Toxicol. Appl. Pharmacol.* **23,** 789–791.

TANFORD, C. (1973). "The Hydrophobic Effect." Wiley, New York.

TRUDELL, J. R., BÖSTERLING, B., and TREVOR. A. J. (1982). Reductive metabolism of carbon tetrachloride by human cytochromes P-450 reconstituted in phospholipid vesicles: Mass spectra identification of trichloromethyl radical bound to dioleoyl phosphatidylcholine. *Proc. Natl. Acad. Sci. USA* **79,** 2678–2682.

TYSON, C. A., HAWK-PRATHER, K., STORY, K. L., and GOULD, D. H. (1983). Correlations of *in vitro* and *in vivo* hepatotoxicity for five haloalkanes. *Toxicol. Appl. Pharmacol.* **70,** 289–302.