Dependence of the Carbon-Tetrachloride–Induced Death of Cultured Hepatocytes on the Extracellular Calcium Concentration

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The role of extracellular Ca^{2+} ions in the killing of liver cells by CCl_4 was studied in primary cultures of rat hepatocytes. The dependence of *in vitro* cell killing on the metabolism of CCl_4 was first examined in order to document the similarity between the action of CCl_4 on cultured hepatocytes and the action of CCl_4 on liver cells in the intact animal. Cells prepared from male rats pretreated with phenobarbital were more sensitive to CCl_4 than cells prepared from either male or female rats. The killing of hepatocytes by CCl_4 was prevented by addition of SKF 525A to the culture medium. This protection was accompanied by evidence of decreased CCl_4 metabolism as assessed by the extent of covalent binding of ^{14}C-CCl_4 metabolites to total cellular lipids and proteins, and by the extent of formation of conjugated dienes accompanying the peroxidation of phospholipids isolated from total cell lipids. The extent of killing of the hepatocytes by CCl_4 was dependent on the Ca^{2+} concentration in the tissue culture medium. Total Ca^{2+} concentrations lower than 0.10 mM were not associated with any CCl_4-induced cell death, and the number of dead cells increased with increasing Ca^{2+} from 0.3 to 3.6 mM. This dependency on extracellular Ca^{2+} was not due to dependency of the extent of metabolism of CCl_4 on Ca^{2+}. The Ca^{2+} concentration in the medium had no effect on the extent of covalent binding of metabolites of CCl_4 to lipids and to proteins and on the extent of peroxidation of phospholipids as shown by the formation of conjugated dienes. In addition, hepatocytes incubated in low Ca^{2+} with CCl_4 developed further evidence of cell injury, as indicated by the killing of these cells following the addition of high Ca^{2+} concentrations under conditions prohibiting any further metabolism of the CCl_4. The results of this study indicate that it is the presence of extracellular Ca^{2+} that converts initially nonlethal cell injury into irreversible cell injury in CCl_4-treated cells. This action of Ca^{2+} most likely represents an influx into the cell across an injured permeability barrier at the plasma membrane, in accord with the accumulation of large quantities of Ca^{2+} in CCl_4-intoxicated liver cells in the intact animal. The relation between this alteration in Ca^{2+} homeostasis and the metabolism of CCl_4 is discussed. (Am J Pathol 1981, 105:138–148)

RECENT STUDIES IN our laboratory have revealed an important role for extracellular calcium ions in the cell death produced by certain chemical[1,2] and biological toxins,[3] mineral particles,[4] and ischemia.[5–7] In each case, the cells seemed to be injured through interaction with the particular agent in an initial series of events that did not necessarily require extracellular Ca^{2+}. However, the consequence of this interaction resulted in disruption of the plasma membrane permeability barrier to Ca^{2+} ions. All cells in the body are bathed in a fluid very rich in Ca^{2+} ions, while intracellular Ca^{2+} concentrations are very much lower. The large resulting gradient is maintained by the relative impermeability of the plasma membrane and by active extrusion of Ca^{2+}. Disruption of this permeability barrier would result in an influx of Ca^{2+}, which would then represent a final common pathway mediating the death of the cells.

That this scheme is relevant to the mechanisms of cell injury that occur with a variety of chemicals is suggested by the accumulation of Ca^{2+} ions produced by a number of distinct hepatotoxins.[8] Probably the best studied of these is CCl_4. Liver cells lethally injured by CCl_4 accumulate large amounts of Ca^{2+}.[9–12] While it is reasonable to assume that this accumulation is causally related to the liver cell death, it remains possible that the appearance of large concen-

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trations of intracellular Ca^{2+} in CCl_4-intoxicated cells simply represents the passive equilibration of Ca^{2+} concentrations in cells lethally injured by different and as yet unexplained mechanisms.

In order to explore more directly the role of Ca^{2+} ions in CCl_4-induced liver cell death, we have examined the dependence on extracellular Ca^{2+} of the killing by CCl_4 of cultured rat hepatocytes. Conditions are described in which the cell death produced by CCl_4 reproduces many of the features of the action of this toxin on liver cells in the intact animal. Under such conditions, the extent of liver cell death is dependent on the Ca^{2+} concentration in the culture medium. This dependency cannot be explained by a similar relationship of the metabolism of CCl_4 to the extracellular Ca^{2+} concentration. Rather, it is concluded that it is the presence of extracellular Ca^{2+} that makes initially nonlethal cell injury irreversible. These data imply that the accumulations of Ca^{2+} in CCl_4-intoxicated liver cells in the intact animal are related in a similar manner to the appearance of cell death.

Materials and Methods

Female and male Wistar (150–200 g) and male Sprague-Dawley rats (150–200 g) were obtained from Charles River Breeders. Male rats pretreated with phenobarbital were given 80 mg/kg sodium phenobarbital intraperitoneally for 3 days. All animals were fasted overnight prior to use.

Isolated hepatocytes were prepared by collagenase perfusion as described previously.[1] Yields of 2–4 $\times$ 10^8 cells/liver with 90% viability (trypan blue exclusion) were routinely obtained. The hepatocytes were plated in either plastic 25-sq cm flasks (Corning) at a density of 12,000 cells/sq cm or in 75-sq cm flasks (Corning) at a density of 67,000 cells/sq cm in Williams' E medium (Flow Laboratories) containing 100 mM Hepes buffer, pH 7.4, 10% heat inactivated (56 C for 10 minutes) fetal calf serum (Flow Laboratories, Rockville, Md), 50 μg/ml Garamycin (Schering), and 0.02 U/ml insulin. Williams' E plus serum, Garamycin, and insulin is referred to below as complete Williams' medium. After incubation in an humidified atmosphere of 5% CO_2–95% air for 90–120 minutes to allow the attachment of viable cells, the cultures were rinsed once with prewarmed Hanks' balanced salt solution (Flow Laboratories) to remove unattached dead cells. The cultures were then incubated in complete Williams' medium with the additions indicated below. In the experiments specifically considering the role of extracellular Ca^{2+} ions, the hepatocytes were initially incubated for 120 minutes and then rinsed 3 times with prewarmed Ca^{2+}-free Hanks' balanced salt solution (Flow Laboratories). They were then placed in Williams' E that was prepared without any added $CaCl_2$ (Flow Laboratories) and that contained 5% fetal calf serum, Garamycin and insulin.

CCl_4 (Fisher Scientific, Pittsburgh, Pa) was added to the cells as a 5% solution in absolute ethanol. Control flasks were prepared both with and without addition of an equal volume of ethanol. SKF 525A was obtained from the Smith Kline & French Laboratories (Philadelphia, Pa). It was dissolved in culture medium and added to the cells at the doses indicated in the text. Cell viability was assayed by trypan blue exclusion as described previously.[1] Viability is expressed as the percentage of the number of unstained, attached cells after the initial 90–120 minute incubation. Unattached cells released from the plastic surface failed to exclude trypan blue at all times. All measurements were made by counting 5 fields each of triplicate cultures.

The covalent binding of ^{14}C-CCl_4 metabolites to protein and lipids was measured after incubating hepatocytes with a 5% solution in ethanol of ^{14}C-CCl_4 (New England Nuclear, Boston, Mass). The total radioactivity in each experiment is specified in the text. The incubation was for 1 hour. Preliminary experiments revealed that there was no appreciable increase in the amount of covalent binding after 1 hour. At the end of the incubation, the medium was aspirated and 5 ml of 10% trichloroacetic acid were added to determine binding to proteins. The hepatocytes were scraped, the suspension was recovered by centrifugation, and the proteins were prepared according to Rao and Recknagel.[13] The dry protein residue was weighed in tared scintillation vials, dissolved in 1 ml of NCS tissue solubilizer (Amersham, Arlington Heights, Ill), and counted in 15 ml of a toluene-based solution. To measure the binding of ^{14}C-CCl_4 metabolites to lipids, the incubation medium was aspirated and 4 ml of methanol were added. The cells were scraped into this solution. Two volumes of chloroform were added to the methanol suspension of hepatocytes and the lipids were extracted according to Folch et al.[14] The number of total lipids in the choloroform layer was determined according to Chiang et al.[15] Aliquots of the lipid solutions in chloroform were added directly to scintillation vials and dried. The lipids were redissolved in 10 ml of a toluene-based scintillation fluid.

Conjugated dienes were determined on total lipid extracts or on phospholipids isolated from them. Total lipids were extracted as described above. An aliquot was used for determination of lipid content.

Another aliquot was evaporated at 37 C under a stream of oxygen-free N_2. The dried lipids were dissolved in cyclohexane to a concentration of 0.5 mg/ml. Spectrophotometric examination over the ultraviolet range of the lipids in cyclohexane was carred out in a Shimadzu Spectronic 210 UV spectrophotometer. Phospholipids were precipitated from the original total lipid solution according to Borgström.[16] The precipitate was resuspended in 15 ml of chloroform–methanol (2:1, v:v). Three ml of water were added, and the phospholipids were recovered in the chloroform phase. Phospholipids were quantitated according to Harris and Popat.[17] To determine the content of conjugated dienes, an aliquot of the chloroform phase was evaporated under oxygen-free N_2, and the phospholipids were resuspended in cyclohexane and analyzed as above.

Results

CCl_4 Killing of Cultured Hepatocytes

When placed in culture, isolated rat hepatocytes readily attach to the surface of plastic dishes and remain viable for at least 3–4 days. Trypan blue exclusion was used throughout to assess viability. We have shown previously[1,4] that this assay correlates very closely with other measures of the viability of cultured cells. After an initial 1.5–2 hours to allow for cell attachment, cultures were treated with 0.35 μl/ml CCl_4. Hepatocytes prepared from either male or female rats rapidly lost viability with over 90% of the cells dead within 4 hours (Table 1). However, before these data could form the basis of an *in vitro* system to study the mechanisms of CCl_4-induced liver cell death, it was essential to consider whether such killing of the cells was likely to be the result of mechanisms similar to those responsible for the toxicity of CCl_4 in the intact animal. For, while the use of cultured cells provides the obvious advantage that the extracellular environment can be more readily manipulated (in particular to remove Ca^{2+} ions from the extracellular fluid), this advantage carries, at the same time, the risk of creating an experimental system that does not reflect events that occur in the intact animal.

Table 1—Killing of Hepatocytes by CCl_4*

Treatment	% Viable hepatocytes
Male rats	
Controls	100 ± 3.6
CCl_4	8.8 ± 0.8
Female rats	
Controls	100 ± 4.7
CCl_4	7.2 ± 3.2

* Isolated hepatocytes were prepared from male or female Wistar rats and allowed to attach in culture for 90 minutes. At this time fresh medium was added containing either 0.35 μl/ml CCl_4 or volume of ethanol equivalent to that added with the CCl_4. Viability of the cells was assessed 4 hours later as the ability to exclude trypan blue. Results are the mean ± SD of three separate flasks.

Table 2—Phenobarbital Pretreatment Enhances Toxicity of CCl_4*

	% Viable hepatocytes
Phenobarbital-treated male rats	41.4 ± 4.3
Male rats	78.4 ± 2.9
Female rats	89.6 ± 6.1

* Isolated hepatocytes were prepared from male Wistar rats pretreated with 80 mg/kg sodium phenobarbital for three days. The cells were allowed to attach in culture for 90 minutes, at which time fresh medium was added that contained either 0.25 μl/ml CCl_4 or a volume of ethanol equivalent to that added with the CCl_4. Viability of the cells was assessed 8 hours later as the ability to exclude trypan blue. Results are the mean ± SD of three separate flasks.

It is generally agreed that, *in vivo,* the toxicity of CCl_4 is dependent upon its metabolism by the NADPH-cytochrome P-450 enzyme system of the liver cell endoplasmic reticulum.[18] Metabolism of CCl_4 is accompanied by the covalent binding of metabolites of CCl_4 to cellular macromolecules and by the peroxidation of phospholipids of the endoplasmic reticular membranes.[18] The dependency of toxicity on CCl_4 metabolism and the biochemical consequences of this metabolism were used to assess the similarities between the toxicity of CCl_4 *in vitro* and *in vivo*.

Dependency of CCl_4 killing on Metabolism

The ability to metabolize CCl_4 and, therefore, the accompanying liver cell injury is enhanced by pretreating rats with an inducer of mixed function oxidase activity, such as phenobarbital. The data in Table 2 indicate that cultured hepatocytes prepared from phenobarbital-pretreated rats are similarly more sensitive to CCl_4 than are cells from uninduced male or female animals. In this case, a lower dose of CCl_4 than that described in Table 1, with only a very slight effect on hepatocytes from male rats, produced considerable killing of hepatocytes from phenobarbital pretreated male rats (Table 2).

This enhanced sensitivity to CCl_4 suggested that killing of cultured hepatocytes by CCl_4 is dependent on the metabolism of the toxin by the mixed function oxidase system. To further document this dependence, an agent was employed that is known to inhibit this metabolism both in intact cells *in vivo* and in isolated microsomes *in vitro*. SKF 525A can prevent the liver cell death produced by CCl_4 in the intact animal[18] as well as inhibit the metabolism of CCl_4 by

Table 3—Prevention by SKF 525A of CCl_4-Induced Liver Cell Death*

Treatment	% Viable hepatocytes
SKF 525A	101.2 ± 2.6
CCl_4	7.8 ± 7.7
CCl_4 plus SKF 525A	105.0 ± 10.3

* Isolated hepatocytes from Sprague-Dawley male rats were incubated for 90 minutes to allow attachment of viable cells. Fresh incubation medium was added either with or without 0.3 μM SKF 525A. After an additional 60 minutes, CCl_4 as a 5% solution in ethanol was added to a final concentration of 0.25 μl/ml. An equivalent amount of ethanol was added to control flasks containing SKF 525A. Viability was measured by trypan blue exclusion 3 hours after addition of CCl_4. The results are the mean ± SD of three separate flasks.

isolated liver microsomes *in vitro*.[19] The ability of SKF 525A to prevent the killing of cultured hepatocytes by CCl_4 is indicated in Table 3. In this case, 0.25 μl/ml CCl_4 killed over 90% of the hepatocytes within 3 hours. Addition of 0.3 μM SKF 525A to the culture medium 1 hour prior to exposing the cells to CCl_4 prevented the killing of the hepatocytes. This action of SKF 525A was a constant result with every preparation of hepatocytes, even though the effective dose would vary from 0.3 to 1 μM. There was also variation in the sensitivity to CCl_4 of different preparations of hepatocytes from phenobarbital-induced animals, reflected in the time course of the killing of the cells. The data shown in Table 3 are from an experiment in which the cells were very sensitive to the CCl_4 with almost all the cells dead by 3 hours. However, even under these conditions of marked toxicity, SKF 525A was an effective inhibitor of the killing of the hepatocytes by CCl_4

Prevention of cell death by SKF 525A was shown to be accompanied by evidence of a decreased metabolism of CCl_4. SKF 525A reduced the extent of covalent binding of CCl_4 metabolites to cellular macromolecules and the extent of the peroxidation of phospholipids. These studies required more cells than those used above, in which viability was the only end point assessed. Freshly isolated hepatocytes were, therefore, plated in 75-sq cm rather than 25-sq cm plastic flasks, and the number of cells plated was increased from 12,000 to 67,000 cells/sq cm. Under these conditions, it was necessary to use somewhat higher doses of SKF 525A than those employed above (Table 3). The optimum concentration was found to be 10 μM. It was also difficult to obtain as complete protection of the cells with SKF 525A as was seen with cells plated in the smaller flasks at the lower density.

Table 4 illustrates a representative experiment in which the effect of SKF 525A on hepatocyte viability and metabolite binding was compared. The data document the extent of covalent binding of ^{14}C-CCl_4 metabolites to whole cell lipids and proteins and the effect of SKF 525A of this binding. Preliminary experiments revealed that such metabolite binding was maximal within 1 hour of the additional ^{14}C-CCl_4 and changed very little thereafter. The data in Table 4, therefore, indicate the metabolite binding measured 1 hour after addition of 0.25 μl/ml ^{14}C-CCl_4. Binding to both lipids and proteins could be readily detected. The binding to lipids was five times greater on a per weight basis than the binding to proteins. This data agrees with reports of others on the extent of lipid and protein binding to microsomes *in* vivo and *in vitro*.[13,20] Addition of 10 μM SKF 525A to the culture medium 30 minutes before exposure of the cells to CCl_4 reduced the extent of covalent binding to both lipids and proteins (Table 4). The covalent binding of ^{14}C-CCl_4 metabolites to lipids was reduced by 38% and to proteins was reduced by 36%. These decreases in metabolite binding paralleled a protection against the cell death by SKF 525A. Over the course of the 10 hours that the cells were observed, 72% of the cells were killed by CCl_4. At the end of 10 hours, however, only 33% of the cells had been killed by the same dose of CCl_4 in the presence of 10 μM SKF 525A. The reduction in CCl_4 metabolism that is implied by the decreases in the extent of metabolite binding to lipids and proteins shown in Table 4 can account for the protection against the cell death observed at the same time. As shown by the data in Figure 1, very small differences in CCl_4 dose and, therefore, presumably in the extent of CCl_4 that is ac-

Table 4—Effect of SKF 525A on Covalent Binding of ^{14}C-CCl_4 Metabolites to Lipids and Proteins*

Treatment	nmoles ^{14}C-CCl_4 bound/mg lipid	% Decrease	nmoles ^{14}C-CCl_4 bound/mg protein	% Decrease
CCl_4	8.89 ± 0.97	—	1.82 ± 0.08	—
CCl_4 plus SKF 525A	5.48 ± 0.77	38	1.16 ± 0.03	36

* Hepatocytes were prepared from Sprague-Dawley males pretreated with 80 mg/kg phenobarbital for 3 days. Cells were plated in 75-sq cm plastic dishes at 67,000 cells/sq cm and incubated for 90 minutes to allow attachment of viable cells. Fresh medium was added either with or without 10 μM SKS 525A. After 30 minutes, 0.25 μl/ml ^{14}C-CCl_4 (10.6×10^5 DPM/ml) was added to all cultures. Covalent binding to total cellular lipids and proteins was determined after 1 hour as described in Materials and Methods. Results are the mean ± SD of three separate flasks.

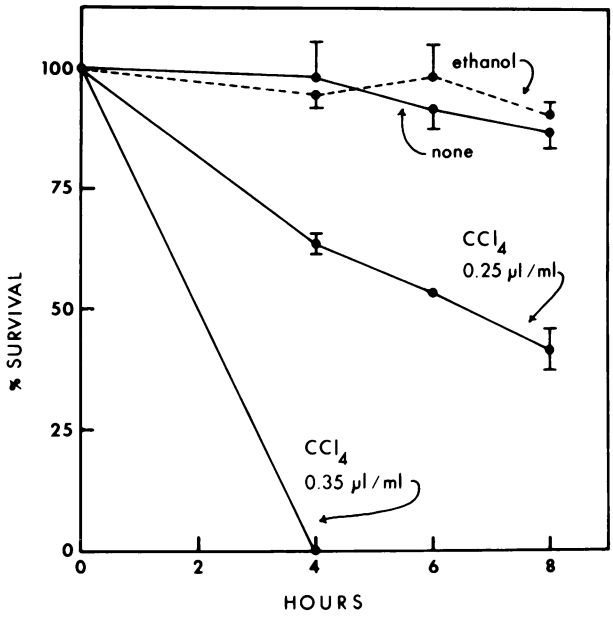

Figure 1—Dose and time dependence of CCl_4-induced death of cultured hepatocytes. Hepatocytes were prepared from Sprague-Dawley rats pretreated with 80 mg/kg phenobarbital for 3 days and plated in 25-sq cm plastic dishes. After an initial 120 minutes incubation, fresh medium was added containing 0.25 µl/ml CCl_4 as a 5% solution in ethanol. Controls received an equal volume of ethanol. Viability was determined at the times indicated by trypan blue exclusion. Results are the mean SD of three flasks.

tually metabolized cause very significant differences in the extent of cell death.

SKF 525A also reduced the extent of lipid peroxidation induced by CCl_4 metabolism. Lipid peroxidation can be detected and quantitated by measurement of ethane or malondialdehyde production or by the detection of conjugated dienes in cellular phospholipids as absorption bands at 230–240 nm.[21] Measurement of ethane production is clearly unsuited to the conditions of tissue culture. Malondialdehyde (MDA) formation has been regularly used to detect the peroxidation *in vitro* of lipids of microsomal membranes or of membranes of other subcellular fractions. However, MDA formation has not been a particularly sensitive assay for lipid peroxidation *in vivo*[22–25] presumably because of the ability of intact cells to metabolize MDA. Preliminary studies of malondialdehyde formation in primary hepatocyte cultures exposed to CCl_4 indicated that only very low levels were detectable. These results agree with the experience in the intact animal (data not shown). Such small amounts of MDA would have been difficult to work with, particularly since the anticipated experiments would have required that even lower levels than those seen with CCl_4 alone be readily measurable.

In contrast, the appearance of conjugated dienes in cellular phospholipids was a sensitive indication of the peroxidation of lipids induced by CCl_4 in the cultured hepatocytes. Previous attempts to relate the toxic injury of isolated hepatocytes to the extent of the peroxidation of phospholipids (assessed by the appearance of conjugated dienes) were based on the analysis of total lipid extracts and had only limited success.[26,27] It was not possible under the conditions of the studies reported here to detect a conjugated diene absorption spectrum (ie, absorption bands at 230–240 nm) in total lipid extracts of the cells. In every case, there was a broad band of absorbent material with a maximum at about 272 nm. This band was highest in the total lipids derived from control cells, lower in total lipids from ethanol-treated controls, and still lower in lipids derived from CCl^4-treated cells (data not shown). The presence of 10 µM SKF 525A lowered the concentration of such absorbent material. These data imply the presence of some

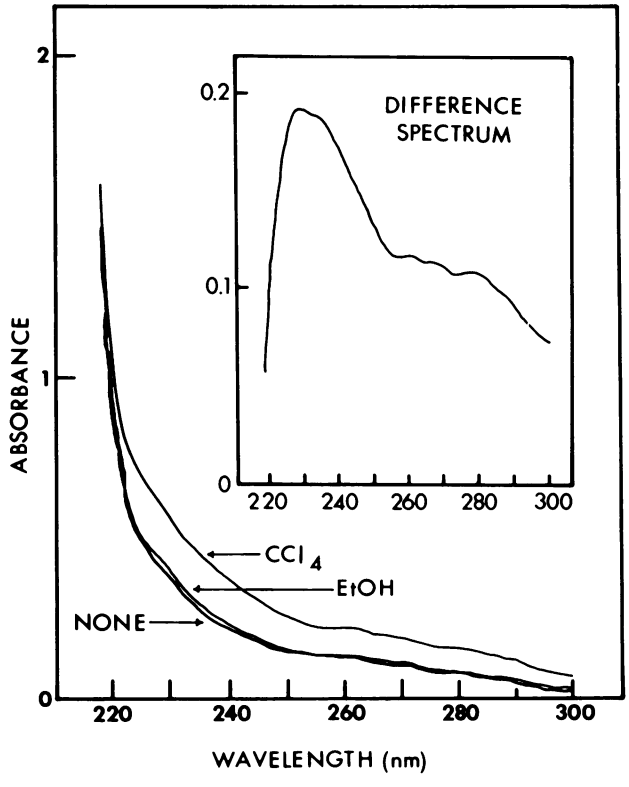

Figure 2—Effect of CCl_4 on absorption spectrum of total hepatocyte phospholipids. Hepatocytes were prepared from male Sprague-Dawley rats pretreated with 80 mg/kg sodium phenobarbital for 3 days and plates in 75-sq cm plastic dishes. After an initial 90 minutes incubation, fresh medium was added to the cells. After an additional 30 minutes, CCl_4 as a 5% solution in ethanol was added to a final concentration of 0.25 µl/ml. Controls were either treated with an equal volume of ethanol or received no further addition. Total cellular lipids were extracted after 1 hour and phospholipids were isolated from these extracts. The phospholipid concentration used to obtain the spectrums illustrated was 0.5 mg/ml cyclohexane in each case. **Inset**—Difference spectrum between the phospholipids from the CCl_4-treated cells and the untreated controls.

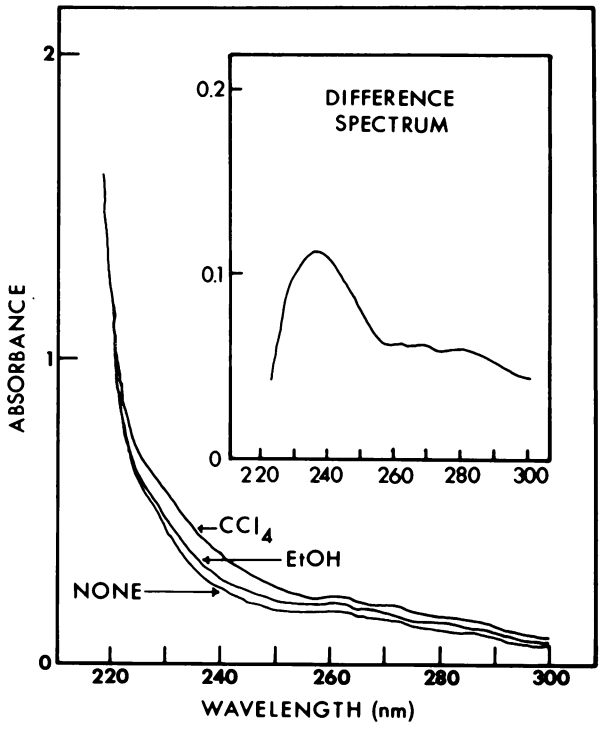

Figure 3—Reduction by SKF 525A in the extent of formation of conjugated dienes in CCl_4-treated hepatocytes. Conditions were identical to those of Figure 2. In this case, all of the hepatocytes were treated with 10 μM SKF 525A. The phospholipid concentration used to obtain the spectrums illustrated was 0.5 mg/ml cyclohexane. **Inset**—Difference spectrum between the phospholipids from CCl_4-exposed cells and the unexposed controls.

lipidlike substance in the cultures that depended on mixed function oxidase activity and the absorption spectrum of which prevented the determination of conjugated dienes.

A pure conjugated diene spectrum, however, could be obtained by simply separating the phospholipids from the total lipids. The relatively fast acetone precipitation method[16] was used to avoid longer preparation procedures that might permit oxidative decomposition of the phospholipids. With this technique, 98% of the phospholipids in the total lipid extracts were recovered. Figures 2 and 3 document that typical conjugated diene spectrums were observed with the isolated phospholipids. Figure 2 shows the spectrums of the lipids prepared from cells treated with CCl_4 without SKF 525A, and Figure 3 shows the spectrums of the phospholipids from cells treated with CCl_4 and SKF 525A. CCl_4 treatment produced a very significant appearance of conjugated dienes in total cell phospholipids when compared with untreated cells or cells exposed to ethanol alone (Figure 2). The inset in Figure 2 is the difference spectrum between the total phospholipids from control cells and those from CCl_4-treated cells. The maximum at 230–240 nm is typical of conjugated dienes and indicates the presence of peroxidized lipids in the CCl_4-intoxicated cells. By comparing Figures 2 and 3, it can be seen that the conjugated diene absorption caused by CCl_4 intoxication was lowered by about 50% by pretreating the cells with SKF 525A. This parallels the protection of the viability of the cells by the same amount under these conditions.

On the basis of the above data, it can be concluded that the CCl_4-induced death of cultured hepatocytes resembles in several important ways the action of CCl_4 on liver cells in the intact animal. The extent of killing *in vitro* was related to the extent of the metabolism of CCl_4 as assessed by the relationship to mixed function oxidase levels and by the protective action of SKF 525A. As *in vivo,* the extent of killing was correlated with the amount of covalent binding of ^{14}C-CCl_4 metabolites to lipids and proteins and with the peroxidation of phospholipids.

Extracellular Ca^{2+} and CCl_4-Induced Liver Cell Death

The relation between the concentration of Ca^{2+} ions in the tissue culture medium and the killing of hepatocytes by CCl_4 was determined by incubating these cells with CCl_4 and increasing concentrations of Ca^{2+}. After an initial period of attachment of 2 hours in medium containing 1.8 mM $CaCl_2$, the cells were placed in medium in which the only source of Ca^{2+} ions was that added with the 5% fetal calf serum. The Ca^{2+} concentration was 0.065–0.090 mM as determined by atomic absorption spectroscopy and is the "O" point on the abscissa in Figure 4. After 10 hours of exposure to 0.25 μl/ml CCl_4, there was no loss of viability of the cells in this medium. When the cells were exposed to the same concentration of CCl_4 for 10 hours in medium to which increasing amounts of $CaCl_2$ were added, there was a progressive decrease in the number of viable cells (Figure 4). The viability of the control cells was not affected by the various Ca^{2+} concentrations.

This result could not be explained by a dependency of the extent of metabolism of CCl_4 on the $CaCl_2$ concentration in the medium. The covalent binding of ^{14}C-CCl_4 metabolites to cellular lipids and proteins and the peroxidation of cellular phospholipids were again used to assess the metabolism of CCl_4 in hepatocytes exposed to a high and a very low extracellular Ca^{2+} concentration. The extent of the binding of ^{14}C-CCl_4 metabolites to total cellular lipids and proteins was identical whether the cells were incubated in 3.5 mM $CaCl_2$ or in medium without any added Ca^{2+} (0.065 mM $CaCl_2$). The extent of covalent binding

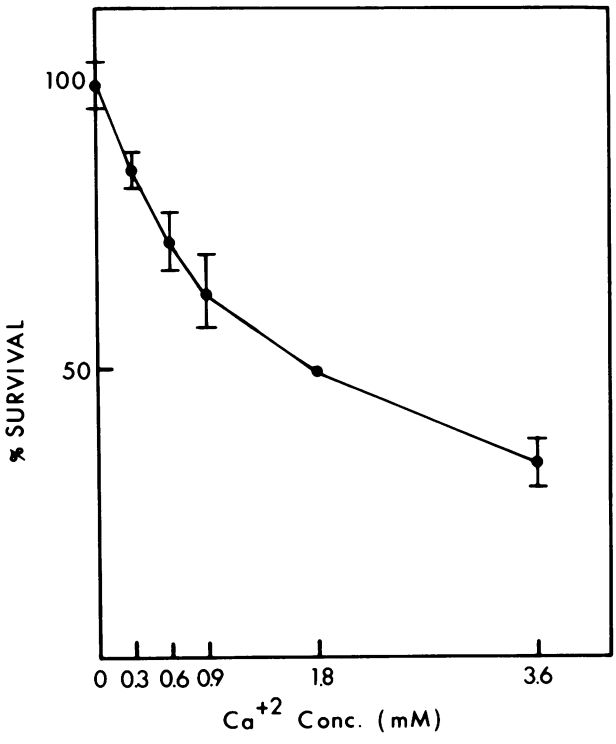

Figure 4—Ca^{2+} dependence of CCl_4-induced killing of cultured hepatocytes. Hepatocytes were prepared from male Sprague-Dawley rats and plated in 25-sq cm plastic dishes. After an initial 120 minutes incubation, fresh Williams' E medium made up without any added Ca^{2+} and containing 5% fetal calf serum, 50 μg/ml garamycin, 0.02 U insulin, and 0.15 μl/ml CCl_4 was added. $CaCl_2$ was added to individual flasks in the concentrations indicated on the ordinate. "O" Ca^{2+} was 0.06 mM and contained that Ca^{2+} added with the serum. All cultures were incubated for 10 hours. Viability was determined by trypan blue exclusion. Results are the mean SD of three flasks.

determined 1 hour after exposure to 0.25 μl/ml CCl_4 was the same in cells incubated in either Ca^{2+} concentration (data not shown). Similarly, Figure 5 indicates that the appearance of conjugated dienes in total cell phospholipids was not dependent on the Ca^{2+} content of the culture medium. The panel on the left (Figure 5A) shows the absorption and difference spectrums of phospholipids isolated from control cells and cells treated with CCl_4 in the presence of 3.6 mM $CaCl_2$. The panel on the right (Figure 5B) shows the spectrums from phospholipids isolated from cells treated with the same concentration of CCl_4 in medium containing no added Ca^{2+} except that present in the 5% fetal calf serum (0.065 mM $CaCl_2$). The extent of lipid peroxidation, as assessed here by the extent of formation of conjugated dienes, was slightly greater in the cells exposed to the "minus calcium" medium.

There are two possible objections to the use of the above evidence to conclude that the dependency of the killing by CCl_4 on the Ca^{2+} concentration of the medium is not a consequence of a dependency of the metabolism of CCl_4 on Ca^{2+}. On the one hand, it might be argued that in the cases of both covalent binding and lipid peroxidation, the extent of metabolism was assessed at much earlier times than that of the effect on viability. It is conceivable that some other manifestation of CCl_4 metabolism related to the Ca^{2+} concentration in the medium develops between these two times. Second, it has not been conclusively established that either the covalent binding of CCl_4 metabolites to cellular macromolecules or the peroxidation of phospholipids necessarily reflects the extent of lethal cellular damage. There might be some other interaction of CCl_4 with the cells that is causally related to the cell death and dependent, in turn, on the Ca^{2+} ion concentration in the medium. The final experiment addressed these considerations.

It was possible to demonstrate that it is the presence of sufficient extracellular Ca^{2+} ions that converts otherwise nonlethal cell injury into irreversible cell injury, measured as the loss of viable hepatocytes. Table 5 summarizes the results of this experiment. Hepatocytes were cultured with 0.15 μl/ml CCl_4 in culture medium containing either 3.6 mM or 0.065 mM $CaCl_2$. After 11 hours, the cells in low calcium were exposed to 3.6 mM $CaCl_2$ for an additional 3 hours. The viability of all cultures was determined at 14 hours. In high calcium for 14 hours, 50% of the cells were dead (line 1). In low calcium for the same time, only 18% of the cells were dead (line 2). However, if the cells were kept in low calcium for the first 11 hours and then exposed to high calcium for 3 hours, 55% of the cells were dead (line 3). That this loss of cells accompanying the late addition of Ca^{2+} ions is the effect of these ions acting on already injured cells is indicated by the inability of the SKF 525A to protect the cells when added at 11 hours (line 4), rather than initially, prior to exposing the cells to CCl_4 (line 5). As a further control, SKF 525A did significantly protect the cells in 3.6 mM $CaCl_2$ when added prior to the start of the first 11 hour incubation (line 6).

Discussion

The major conclusion to be drawn from the above data is that killing of cultured hepatocytes by CCl_4 can be shown to be dependent on the concentration of Ca^{2+} ions in the extracellular environment. Furthermore, this requirement for extracellular Ca^{2+} cannot be attributed simply to a dependence of the metabolism of CCl_4 on Ca^{2+}. Rather, the data indicate that, in the absence of Ca^{2+}, the hepatocytes manifest sufficient evidence of the toxic effect of the metabolism of CCl_4 such that subsequent addition of Ca^{2+} to

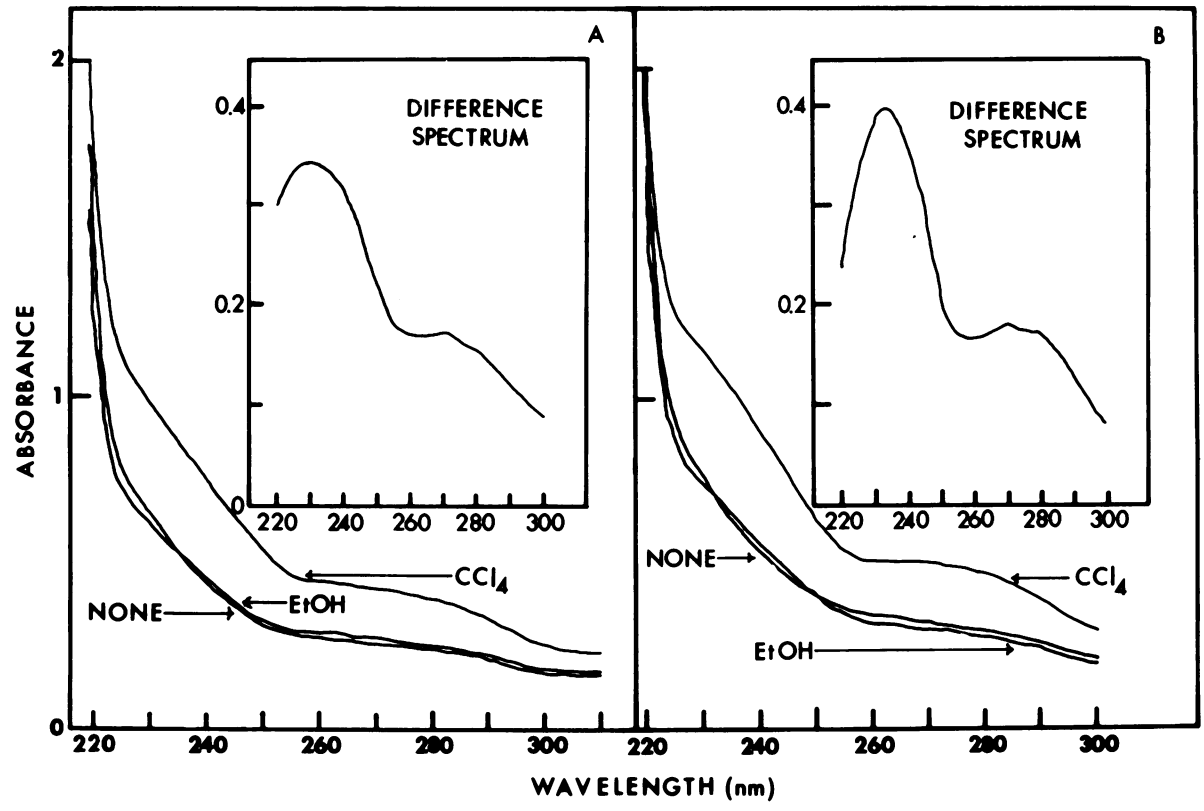

Figure 5—Diene conjugation in whole cell phospholipids from hepatocytes cultured in either high Ca^{2+} (3.6 mM) or low (0.06 mM) Ca^{2+}. Hepatocytes were prepared from male Sprague-Dawley rats pretreated with phenobarbital and plated in 75-sq cm plastic dishes. After an initial incubation for 120 minutes, fresh medium was added containing either 3.6 mM $CaCl_2$ (*A, left panel*) or no additional calcium except that added with the fetal calf serum (*B, left panel*, 0.06 mM Ca^{2+}). To separate flasks containing the high or low Ca^{2+} content were then added either 0.25 μl/ml CCl_4 as a 5% solution in ethanol, ethanol alone, or nothing. After 1 hour incubation, total lipids were extracted from the cells and phospholipids isolated from these lipids. The concentration of phospholipids used to record the absorption spectrums was 1.0 mg/ml cyclohexane. Each spectrum was obtained from the phospholipids prepared by pooling the cells from three flasks. **Insets** illustrate the difference spectrum between the phospholipids from the CCl_4-treated cells and the untreated controls.

the tissue culture medium produces considerable cell death without a requirement for further metabolism of CCl_4. Because of the lethal consequences of an elevated intracellular Ca^{2+} content,[1,28] the simplest interpretation of these findings is that the metabolism of CCl_4 leads to a disruption of normal intracellular calcium homeostasis, as a result of plasma membrane injury. In the presence of sufficient extracellular Ca^{2+}, these ions enter the injured cells at such a rate that they accumulate, with the inevitable consequence of the death of the cells. This conclusion is consistent with previous demonstrations of plasma membrane dysfunction in CCl_4-intoxicated cells, in particular,[29-31] and with previous studies of the relation between plasmalemma injury and toxic cell death, in general.[32-37]

Where the killing of hepatocytes was dependent on the concentration of extracellular Ca^{2+}, the cell death was also dependent on the metabolism of CCl_4. This fact helps relate the conditions of the *in vitro* toxicity of CCl_4 to the conditions *in vivo* because, in the intact animal, liver cell death is dependent on the metabolism of CCl_4.[20] Dependency on CCl_4 metabolism in the killing of cultured hepatocytes was implied by the enhanced sensitivity to CCl_4 of cells from phenobarbital-treated animals (Table 2) and by the protection seen with SKF 525A (Table 3). In parallel with this protection, SKF 525A reduced the extent of metabolism of CCl_4, as reflected in the extent of covalent binding of ^{14}C-CCl_4 metabolites to cellular lipids and proteins (Table 4) and in the extent of peroxidation of phospholipids (Figures 3 and 4).

The connection between the metabolism of CCl_4 and the production of lethal cell injury has not been clearly defined. The initial consequence of the metabolism of CCl_4 is a disruption of the integrity of the endoplasmic reticulum, evident in the loss of cytochrome P-450, glucose-6-phosphatase, and active transport of Ca^{2+} ions. While both covalent binding of CCl_4 metabolites and the peroxidation of membrane phospholipids have been invoked to explain the effect of CCl_4 metabolism, recent evidence indicates that lipid peroxidation is at least responsible for the loss of cytochrome P-450 and glucose-6-phos-

Table 5—Two-Step Killing of Hepatocytes by CCl_4 and Ca^{2+}*

Group	SKF 525A pretreatment (1 μM)	First incubation period (mM $CaCl_2$)	Second incubation period (mM $CaCl_2$)	% Viable hepatocytes
1	–	3.6	3.6	49.6 ± 6.4
2	–	0.06	0.06	81.9 ± 13.3
3	–	0.06	3.6	45.4 ± 6.7
4	–	0.06	3.6 (plus 1 μM SKF 525A)	48.6 ± 6.1
5	+	0.06	3.6 (plus 1 μM SKF 525A)	73.5 ± 12.7
6	+	3.6	3.6	70.0 ± 6.9

* Hepatocytes were prepared from male Sprague-Dawley rats and plated in 25-sq cm plastic dishes at 12,000 cells/sq cm. After a 90 minute incubation, half of the flasks received fresh medium containing 3.6 mM $CaCl_2$ and half received medium containing 0.06 mM $CaCl_2$. All flasks were given CCl_4 (0.15 μl/ml). Where indicated, 1 μM SKF 525A was added 30 minutes before CCl_4. After an 11 hour incubation, the flasks were given, as indicated below, either $CaCl_2$ to 3.6 mM, 1 μM SKF 525A, both, or no further additions. Viability of the cells in all flasks was determined after an additional 3 hour incubation (14 hours after addition of CCl_4). Results are the mean ± SD of three separate flasks.

phatase.[20] An initial damage to the endoplasmic reticulum can be linked to further events that lead ultimately to an inability to maintain calcium homeostasis with influx and accumulation of these ions. On the one hand, an early rise in total liver cell Ca^{2+} content accompanies the loss of calcium pump activity in isolated microsomes.[9,10] Mitochondria and the endoplasmic reticulum sequester calcium and help to maintain the cytosolic concentration at very low levels[38,39] Loss of calcium sequestration by the endoplasmic reticulum would lead to an elevated cytosolic Ca^{2+} concentration, with more of these ions being taken up by the mitochondria, and an accompanying rise in total calcium content. The rise in cytosolic Ca^{2+} may also activate membrane-bound phospholipases. Loss of phospholipid from the plasma membrane as a result of uncontrolled phospholipase action would, in turn, destroy the calcium permeability barrier.

The early rise in liver Ca^{2+} is only very transient with a peak some twice the normal level at about 2 hours and a prompt return to the control content by at least 4 hours.[9,10] There is no further increase in total Ca^{2+} content until between 8 and 12 hours. The calcium content then rises steadily with the appearance and accumulation of dead cells. It is difficult to attribute the later calcium accumulation to the activation of phospholipases during the period of the earlier increase in Ca^{2+} content, since the latter is only transient, with at least 4–5 hours of a normal cell Ca^{2+} content separating the two.

The peroxidative decomposition of phospholipids release soluble products capable of damaging other membranes separated from the site of lipid peroxidation.[40–45] Among the many compounds released, fatty aldehydes have been particularly implicated in the membrane damage produced.[45–48] It can be argued that the induction of lipid plroxidation at the site of CCl_4 metabolism releases soluble, toxic products that induce membrane damage at sites removed from the original locus of lipid peroxidation. This would account for the delay in which the early manifestations of microsomal membrane injury are followed several hours later by the accumulation of intracellular Ca^{2+} and the appearance of liver cell death.

Evidence of the peroxidation decomposition of phospholipids in cultured hepatocytes treated with CCl_4 was shown to correlate with the cell death (Figures 2 and 3). This is an important observation that serves to relate the mechanisms of the toxicity of CCl_4 in cultured hepatocytes to similar mechanisms in the intact animal. Peroxidation of cellular phospholipids in isolated hepatocytes has been sought previously under a variety of situations generally either by measurement of malondialdehyde production or by formation of conjugated dienes.[26,27,49–56] As noted above, malondialdehyde could not be used as a reliable index of lipid peroxidation in the cultured hepatocytes. On the other hand, investigators have had difficulties using conjugated dienes as a means of detecting and quantitating the extent of lipid peroxidation in isolated hepatocytes.[14,23] A very likely explanation of these difficulties is that total lipid extracts of such cells cannot be used for determination of conjugated dienes. Through isolation of the phospholipid fraction from these total lipid extracts, it was possible to show clearly that CCl_4 induces the peroxidation of cellular phospholipids of cultured hepatocytes and that the extent of this peroxidation correlates with the extent of the toxicity expressed as loss of viability (Figures 2 and 3). The method employed here for detecting lipid peroxidation is simple, gives virtual quantitative recovery of phospholipids, and is very sensitive.

There is another significant feature that distinguishes the present study from previous ones that

have used hepatocytes to study the mechanisms of action of hepatotoxins in general or of CCl_4 in particular. Here, cultured hepatocytes are attached to plastic dishes rather than being placed in suspension culture for much shorter times. The major advantage of the use of cultured hepatocytes is that their viability can be maintained for considerably longer times than can hepatocytes in suspension culture. This allows assessment of the effects of more subtle and physiologic changes in the culture conditions, since cumulative effects on viability can be determined over much longer time periods. This was particularly important with respect to the objectives of the present study. A requirement for extracellular Ca^{2+} ions in the killing of hepatocytes by CCl_4 as shown by the data in Figure 4, was not observed under all circumstances.[57] Conditions exist under which the killing of hepatocytes by CCl_4 is not Ca^{2+} dependent.[57] Under these conditions, the hepatocytes are injured by CCl_4 such that they fail to exclude trypan blue in Ca^{2+} concentration less than 0.09 mM. It is our experience that these conditions do not readily allow demonstration of the same strict correlation between CCl_4 metabolism, SKF 525A action, and cell viability as shown above, where there was Ca^{2+} dependence of CCl_4 cytotoxicity.[57] These conditions are a high ration of cells to medium, with larger concentrations of CCl_4 and shorter periods of incubation.[57] These are also the conditions that generally obtain with suspension culture of hepatocytes. The important observation, however, is that conditions exist under which it is possible to show that the killing of hepatocytes is Ca^{2+} dependent. The fact that these conditions closely reflect those that occur *in vivo* with regard to the relation between CCl_4 metabolism and its toxicity supports the argument that, as in tissue culture, the liver cell death in the intact animal is also calcium dependent and, therefore, related to large accumulations of intracellular Ca^{2+} that are a prominent feature of the reaction to CCl_4

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