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EFFECTS OF OXYGEN DEFICIENCY AND CALCIUM OMISSION ON CARBON TETRACHLORIDE HEPATOTOXICITY IN ISOLATED PERFUSED LIVERS FROM PHENOBARBITAL-PRETREATED RATS

YASUSUKE MASUDA* and YUMI NAKAMURA
Division of Toxicology, Niigata College of Pharmacy, Niigata 950-21, Japan

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Abstract—The effect of oxygen concentration and Ca^{2+} omission on CCl_4-induced hepatotoxicity was studied in a non-recirculating and hemoglobin-free liver perfusion system using phenobarbital-pretreated rats. With 95% O_2-saturated perfusate, infusion of 0.5 mM CCl_4 caused an instantaneous increase of thiobarbituric acid reactive substances (TBA-RS) in the effluent perfusate, accompanied by only a slight leakage of K^+ and lactate dehydrogenase (LDH). $CBrCl_3$ produced a far greater increase in the TBA-RS level, but again with slight K^+ and LDH leakage. With 20% O_2-saturated perfusate, CCl_4 caused a marked LDH leakage, which was preceded by an early and considerable increase in K^+ leakage coupled with Na^+ uptake. Ca^{2+} uptake was initially slight, being enhanced concurrently with the LDH leakage. The TBA-RS level changed biphasically with an initial moderate and a succeeding greater increase coupled with LDH leakage. *N,N'*-Diphenyl-*p*-phenylenediamine and promethazine suppressed the TBA-RS production, but improved neither K^+ nor LDH leakage. Omission of the Ca^{2+} from the perfusate reduced the initial K^+ leakage as well as the later TBA-RS release, and markedly delayed the LDH leakage. In retrograde perfusion under low oxygen supply with Ca^{2+}, CCl_4 produced essentially the same toxic manifestations as those observed in the anterograde perfusion. Hepatocytes of the periportal and pericentral areas were not stained with trypan blue in the antero- and retrograde perfusion systems respectively. Thus, oxygen deficiency, rather than lipid peroxidation by itself, and the essential role of extracellular Ca^{2+} may be important for CCl_4-induced hepatic cell necrosis, in which plasma membrane permeability change may be an early and critical event.

Carbon tetrachloride (CCl_4) is reductively metabolized by the microsomal cytochrome P450-dependent monooxygenase system, to the trichloromethyl free radical ($\cdot CCl_3$) which is further converted to the peroxy radical ($\cdot OOCCl_3$) in the presence of oxygen. These reactive free radical metabolites of CCl_4, in turn, enhance lipid peroxidation of biomembranes and covalently bind to cellular macromolecules, which are thought to trigger degenerative processes in liver cells [1, 2]. However, the relative contributions of lipid peroxidation and covalent binding to hepatic injury, and the mechanisms linking these initial events to final centrilobular cell necrosis are still unclear.

Oxygen is one of the factors that markedly affect CCl_4 hepatotoxicity. Low oxygen partial pressure has been reported to enhance CCl_4 hepatotoxicity in animals [3, 4], in the perfused rat liver [5], and in isolated hepatocytes [6]. It is still controversial, however, whether the enhanced toxicity is due to increased covalent binding of active CCl_4 metabolites [4, 7] or enhanced lipid peroxidation [6, 8].

Disturbances in cellular calcium homeostasis have long been proposed as a cause of CCl_4-induced liver cell necrosis, such as marked hepatic calcium accumulation in CCl_4-intoxicated rats [9–11], impairment of the microsomal Ca^{2+} sequestration mechanism [12, 13] and decrease in plasma membrane Ca^{2+} uptake [14] soon after CCl_4 administration. In cultured hepatocytes, Casini and Farber [15] and Chenery *et al.* [16] reported the extracellular Ca^{2+} dependence of CCl_4-induced cell death, whereas Smith *et al.* [17, 18] reported that extracelular Ca^{2+} is not essential in hepatocyte suspension. It has been suggested that the discrepancy is due to the presence of vitamin E in the medium [19]. Elevation of cytosolic free Ca^{2+} concentration by CCl_4 has also been demonstrated in isolated hepatocytes [20–22].

In the present study, the significance of these two physiologically essential factors in the development of CCl_4-induced hepatic necrosis was examined by using isolated livers from phenobarbital (PB†)-pretreated rats. The perfused liver is well suited for such purposes, since it is easy to control extracellular concentrations of oxygen and Ca^{2+} and also to continuously monitor concentrations of various constituents in the effluent perfusate. Thus, levels of thiobarbituric acid reactive substances (TBA-RS), leakage of lactate dehydrogenase (LDH), and changes of ionic concentrations (K^+, Na^+ and Ca^{2+}) in the perfusate were measured as indices of lipid peroxidation, cell necrosis and plasma membrane

* Correspondence: Yasusuke Masuda, Division of Toxicology, Niigata College of Pharmacy, 5-13-2, Kamishin'ei-cho, Niigata 950-21, Japan.

† Abbreviations: PB, phenobarbital; DPPD, *N,N'*-diphenyl-*p*-phenylenediamine; KHB, Krebs–Henseleit bicarbonate buffer; LDH, lactate dehydrogenase; MDA, malondialdehyde; and TBA-RS, thiobarbituric acid reactive substances.

integrity. PB pretreatment is well known to cause enhancement of CCl_4 hepatotoxicity [23], which may be due to induction of a specific form of cytochrome P450 that activates CCl_4 [24] in the perivenous zone of liver lobules [25]. Therefore, this treatment may be especially useful for the present short-term perfusion experiments, without altering the basic mechanism of action of CCl_4.

METHODS

Animals and treatments. Male, SPF-grade, Sprague–Dawley rats, 80–100 g in weight, were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Japan, and housed in an air-conditioned animal room (temperature 24 ± 1°, humidity 50–60%). Phenobarbital (PB, 0.1%) was administered in drinking water for 5–6 days. Nourished animals of 150–160 g of body weight were used for liver perfusion experiments.

Liver perfusion. Prior to surgery, rats were anesthetized with 60–70 mg/kg, i.p., of pentobarbital, and 1000 units/kg of heparin was injected into the femoral vein. The liver was cannulated at the portal vein and inferior vena cava and isolated according to the basic surgical procedure [26]. Smaller lobes (proc. papillaris, proc. caudatus and lobus dexter) were tied and cut off in order to attain even perfusion among lobes and also to conserve the perfusion medium. The liver was sustained in a vessel containing the perfusion medium, which was placed in a small box warmed with 37°-circulating water. All connecting tubes were insulated to maintain the efficient temperature at 35–36°. The perfusion was regularly conducted in an anterograde, non-recirculating, constant flow rate (25 mL/min) system. Krebs–Henseleit bicarbonate buffer (118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 5.6 mM glucose, saturated with 95% O_2–5% CO_2 or 20% O_2–75% N_2–5% CO_2 gas mixtures at 37°, hereafter referred to as 95% O_2–KHB or 20% O_2–KHB, respectively) was used as the regular perfusion medium. Experiments commenced exactly 30 min after cannulation of the portal vein, which time was defined as 0 min. Usually, no leakage of the perfusate was observed in uninjured anterograde perfused livers. In injured livers, however, the perfusate started to leak with elevation of effluent LDH activity, the leakage rate increasing with time up to 300–500 mL at the end of the perfusion (120 min). The livers swelled to some extent as soon as the perfusion was switched to retrograde and leakage amounted to 0–50 and 300–500 mL at 80 min in uninjured and injured livers respectively.

After the experiments, all livers were infused immediately with 0.2 mM trypan blue for 10 min and then with 1% paraformaldehyde for 6 min [27], weighed and maintained in 10% neutralized formalin for microscopic examination. The trypan blue infusion was essential to confirm even perfusion among the liver lobes. Data from unevenly perfused livers were omitted.

CCl_4 or $CBrCl_3$ solution was diluted in cold KHB (not gassed) to a concentration of 5 mM in a sealed flask by stirring and occasional sonication. This was infused at 10 min (i.e. 40 min after cannulation) for a period of 30 min at a rate of 2.5 mL/min through a warmed coiled tube. A slight possible loss of CCl_4 in the warmed perfusate into a head space of the air-trap tube (inner diameter, 3 mm; height, 30 cm; with a cock at the top), which was placed between the point of CCl_4 infusion and the liver and usually filled with perfusate at the level of about 15 cm, was inevitable in the present perfusion system. *N,N'*-Diphenyl-*p*-phenylenediamine (DPPD) and promethazine, dissolved in dimethyl sulfoxide and KHB respectively, was infused at 0.025 mL/min.

Monitoring and assay of effluent components. The outlet was connected to a thermister, an oxygen electrode (Clark type), ion selective electrodes for Ca^{2+}, K^+ and Na^+ (Orion) or a pH electrode (Metrohm). The effluent perfusate was collected for 15 sec at appropriate times. For calibration of perfusate oxygen concentration, air-saturated water at 37° (0.217 mM O_2) was used as a routine standard and run through the perfusion system. Na^+ and Ca^{2+} concentrations were calibrated by infusing 0.5 to 2.5 mL/min of distilled water into the regular perfusate flow (25 mL/min) in the absence of the liver. For K^+, a concentrated K^+ standard solution was infused. Large changes of K^+ and Ca^{2+} concentrations could be confirmed by other methods: flame photometry and the tetraphenylboron method [28] for K^+ and the orthocresolphthalein complexone method [29] for Ca^{2+}. TBA-RS in the effluent perfusate was measured essentially according to the method of Ernster and Nordenbrand [30] using malondialdehyde (MDA) as the standard. LDH activity was assayed by the reduction of NAD^+ at 25° by the clinical assay method [31]. One unit was defined as 1 μmol of NAD^+ reduced/min/L of the perfusate.

Microscopic examinations. Trypan blue-stained and formaldehyde-fixed livers were cut with a tissue micro-slicer (Dosaka EM, Japan) into 50 μm thick slices, in 0.1 M phosphate buffer (pH 7.4) containing 20% glycerin. Tissue slices were mounted on glass slides and directly examined under low magnifications.

RESULTS

Infusion of CCl_4 and $CBrCl_3$ under high oxygen supply. In the liver perfused with 95% O_2-regular KHB containing 1.3 mM Ca^{2+}, the inlet (portal) and outlet (venous) oxygen concentrations were 0.615 ± 0.021 (SD) and 0.126 ± 0.029 mM, respectively, with a post-fixed liver weight of 6.3 ± 0.5 g (N = 15). In such perfused livers, infusion of 0.5 mM CCl_4 almost instantaneously increased the oxygen uptake, as reported by Thurman *et al.* [32], and the outlet oxygen concentration reached a low of 0.029 ± 0.008 mM, N = 10, within 4 min. The pH of the effluent perfusate decreased by approximately 0.1 within 10 min, accompanied by an approximately 2.5-fold increase in lactic acid concentration (data not shown). Both oxygen concentration and pH recovered very slowly (Fig. 1A, upper traces). The effluent TBA-RS concentration also started to increase soon after CCl_4 infusion, reaching a peak

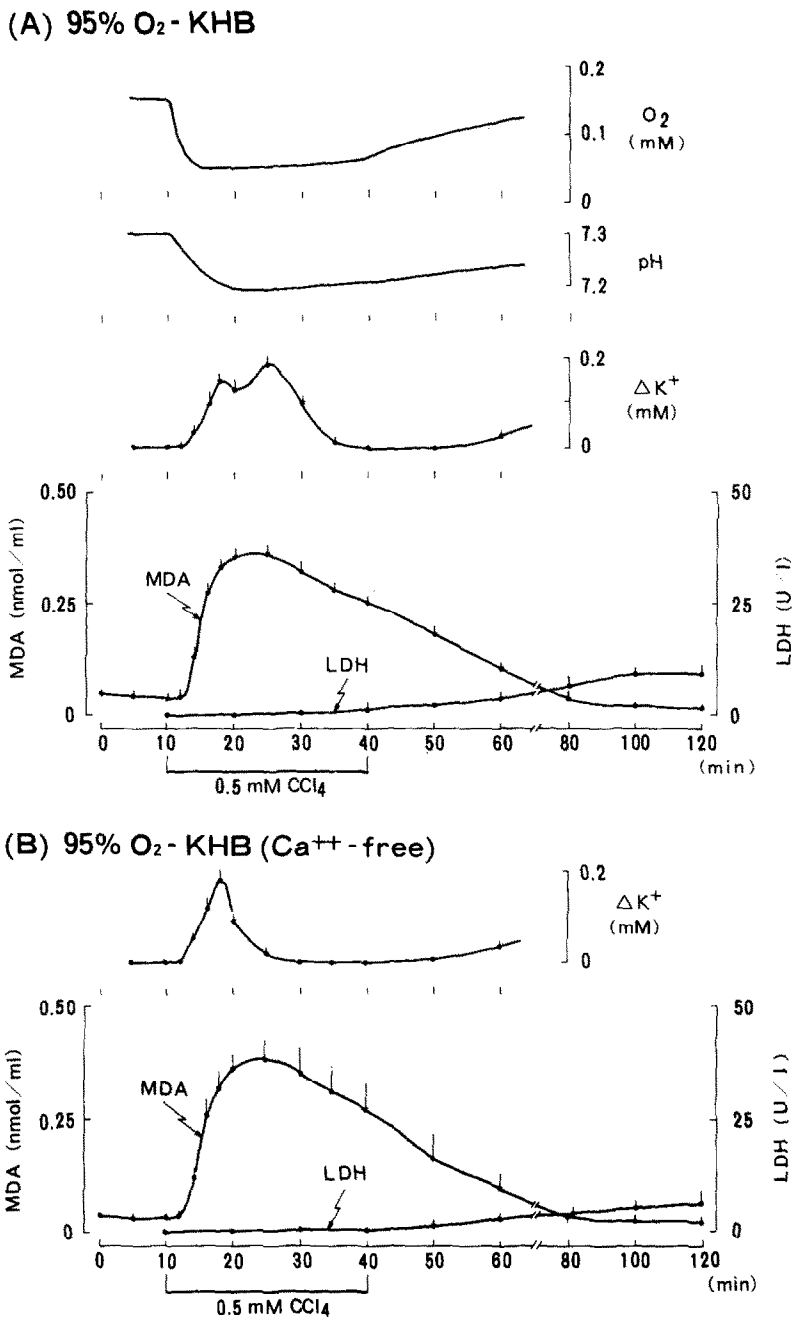

Fig. 1. Leakage of K^+, TBA-RS and LDH following infusion of CCl_4 in the isolated, PB-pretreated rat liver, perfused with 95% O_2-saturated KHB (containing 1.3 mM Ca^{2+}) (A) or Ca^{2+}-free KHB (B). Upper traces are examples of the recordings of oxygen concentration and pH of the effluent perfusate. Values are means ± SE (N = 6 for A and N = 4 for B).

about 15 min later and gradually declining thereafter (Fig. 1A). The perfusate LDH level increased slightly after 40–50 min. An early transient increase in K^+ concentration, approximately 0.2 mM, was observed, but changes in Na^+ and Ca^{2+} concentrations were hardly detectable (data not shown). When Ca^{2+} was omitted from the perfusate, K^+ release and LDH leakage appeared to be decreased slightly, but the pattern and the extent of TBA-RS levels were unchanged (Fig. 1B).

Infusion of the same concentration of $CBrCl_3$ produced a much greater increase in the TBA-RS concentration. LDH leakage was slight such as in the case of CCl_4 infusion. A small biphasic increase in the perfusate K^+ concentration was observed (Fig. 2).

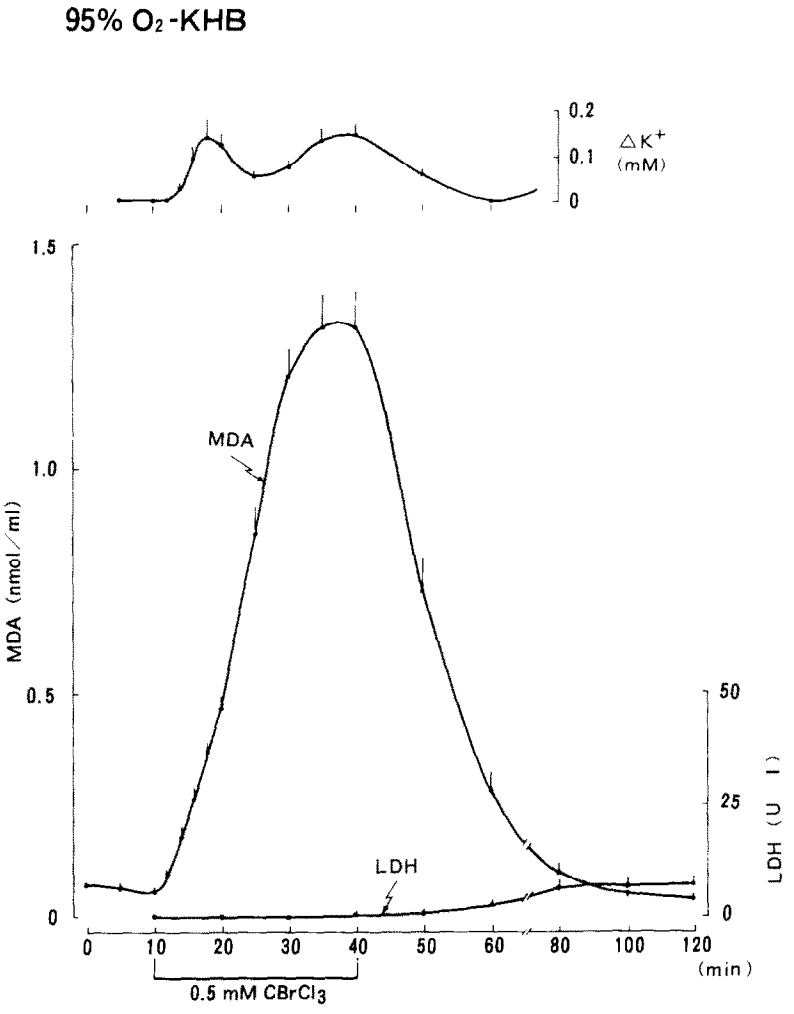

Fig. 2. Leakage of K^+, TBA-RS and LDH following infusion of $CBrCl_3$ in the isolated, PB-pretreated rat liver, perfused with 95% O_2-saturated KHB. Values are means ± SE (N = 6).

Infusion of CCl_4 *under low oxygen supply.* When 20% O_2-KHB was used as the perfusate, inlet and outlet oxygen concentrations were 0.204 ± 0.006 (SD) and 0.023 ± 0.005 mM, N = 14, respectively. The latter value tended to increase slightly up to the time of CCl_4 infusion. In this case, therefore, the pericentral area of the liver lobule was in rather an anoxic state, and further oxygen uptake was almost indiscernible after CCl_4 infusion. In these conditions, CCl_4 infusion produced a marked LDH leakage with a peak 30–35 min later (Fig. 3A). The TBA-RS concentration increased in a biphasic manner: an early moderate increase, which was almost comparable to the case under high oxygen supply, and a succeeding greater increase in parallel with the LDH leakage. The perfusate K^+ concentration markedly increased soon after CCl_4 infusion, preceding the LDH leakage. When Ca^{2+} was omitted from the perfusion medium, LDH leakage was delayed and abruptly started to increase after 100 min (Fig. 4A). The TBA-RS production increased initially, similar to that in the presence of Ca^{2+}, and tended to increase again concurrently with LDH leakage. Initial increases in K^+ concentration were much less, although a gradual increase was observed thereafter.

Without CCl_4 infusion, TBA-RS levels and K^+ concentrations were almost unchanged, and LDH leakage was kept quite low up to 80 min, irrespective of the presence or absence of Ca^{2+} (Fig. 3B and 4B).

Ionic changes in the perfusate after CCl_4 *infusion.* Figure 5 shows an example of simultaneous recordings of the ionic changes following CCl_4 infusion under low oxygen supply. Coupled with the K^+ leakage into the perfusate (ΔK^+_{max} = 1.2 ~ 1.9 mM), a similar Na^+ uptake (ΔNa^+_{max} = −1.0 ~ −1.5 mM) was observed. Both of these changes had almost terminated before LDH leakage started. Ca^{2+} uptake occurred in a biphasic manner; an initial small change coupled with K^+ release was followed by a greater uptake coupled with LDH

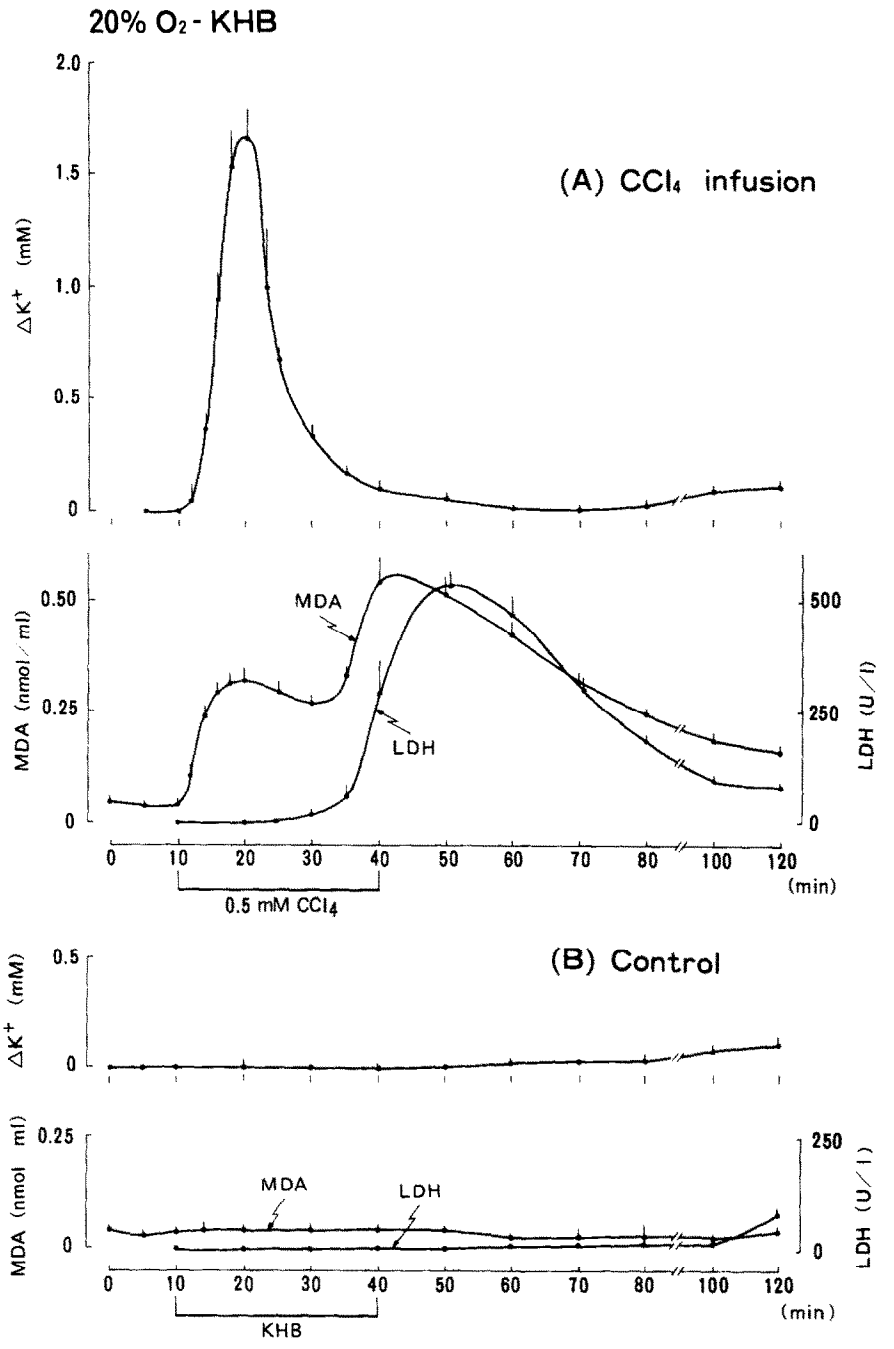

Fig. 3. Leakage of K^+, TBA-RS and LDH following infusion of CCl_4 in the isolated, PB-pretreated rat liver, perfused with 20% O_2-saturated KHB. Values are means ± SE (N = 8 for A and N = 5 for B).

leakage. The time and extent of maximum Ca^{2+} change ($\Delta Ca^{2+}_{max} = -0.06 \sim -0.09$ mM) tended to vary in parallel with those of the LDH leakage.

Effect of DPPD and promethazine. As shown in Fig. 6, under simultaneous infusion of CCl_4 with DPPD (10^{-5} M) or promethazine (10^{-4} M), the TBA-RS production was lowered considerably, whereas increases of K^+ and LDH leakage were not suppressed.

Retrograde perfusion. In the liver perfused in a retrograde manner with 20% O_2-KHB, CCl_4 infusion produced patterns of changes essentially similar to those observed with the anterograde perfusion system, although the changes were greater in the retrograde system (Fig. 7). Little LDH leakage was observed in control livers without CCl_4 infusion.

Microscopic observations. In livers perfused with 95% O_2-KHB and with CCl_4 or $CBrCl_3$, none or

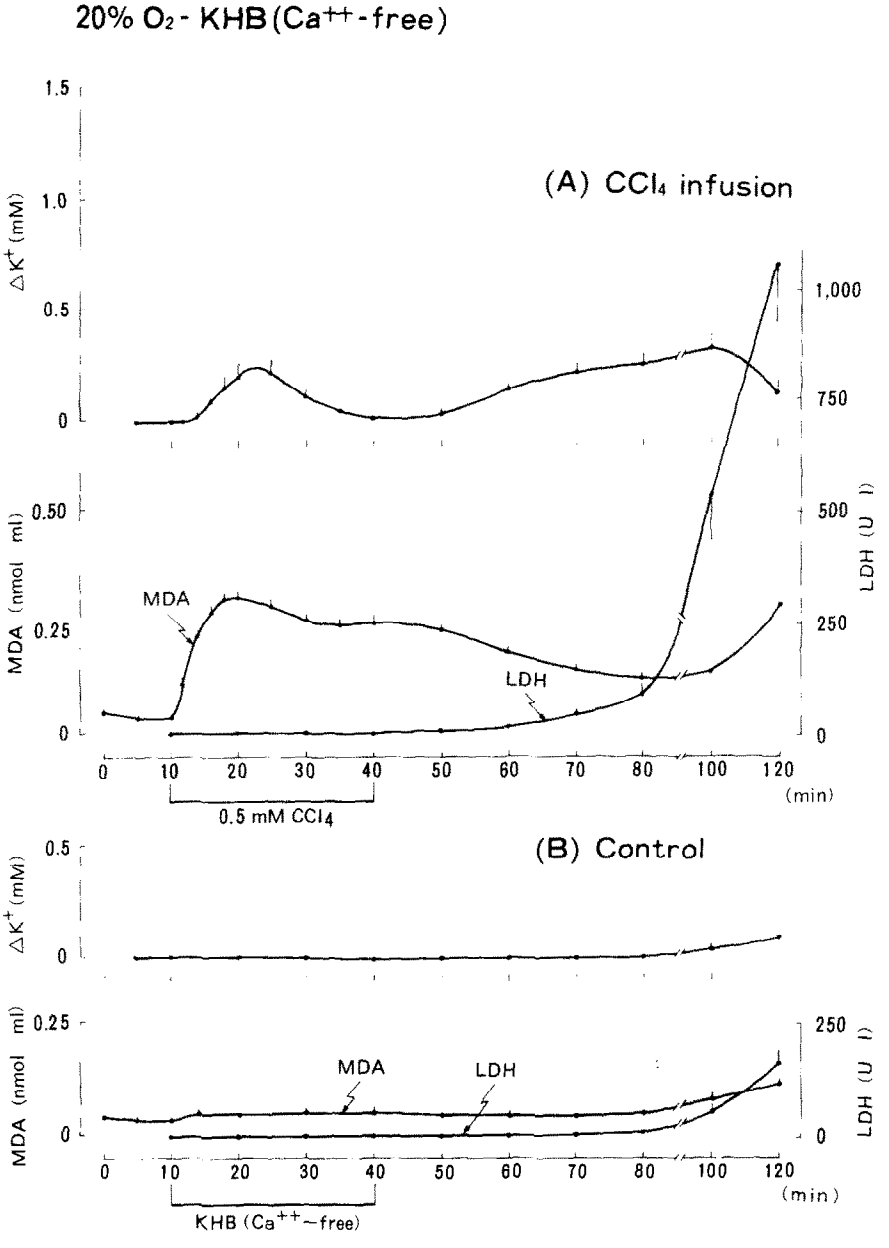

Fig. 4. Leakage of K^+, TBA-RS and LDH following infusion of CCl_4 in the isolated, PB-pretreated rat liver, perfused with 20% O_2-saturated Ca^{2+}-free KHB. Values are means ± SE (N = 8 for A and N = 5 for B).

few hepatocytes were stained with trypan blue after a perfusion period of 120 min (Fig. 8A), in agreement with very slight LDH leakage. In livers perfused with 20% O_2-KHB containing 1.3 mM Ca^{2+}, CCl_4 caused a marked trypan blue uptake in midzonal to pericentral hepatocytes at 80 min, but trypan blue uptake was not observed in periportal cells (Fig. 8B). In the absence of Ca^{2+}, however, little or no trypan blue uptake was observed at 80 min (Fig. 8C), although variable uptake was observed at 120 min. In the retrograde perfusion system with 20% O_2-KHB, CCl_4 infusion caused a marked trypan blue uptake from midzonal to periportal hepatocytes, whereas pericentral cells were not stained at all (Fig. 8D). Through these examinations, midzonal hepatocytes appeared to be more sensitive, which has been pointed out under anoxic perfusion by Marotto *et al.* [33].

DISCUSSION

Using a hemoglobin-free, non-recirculating liver perfusion system, it was shown that infusion of CCl_4 caused an early elevation of TBA-RS in the effluent

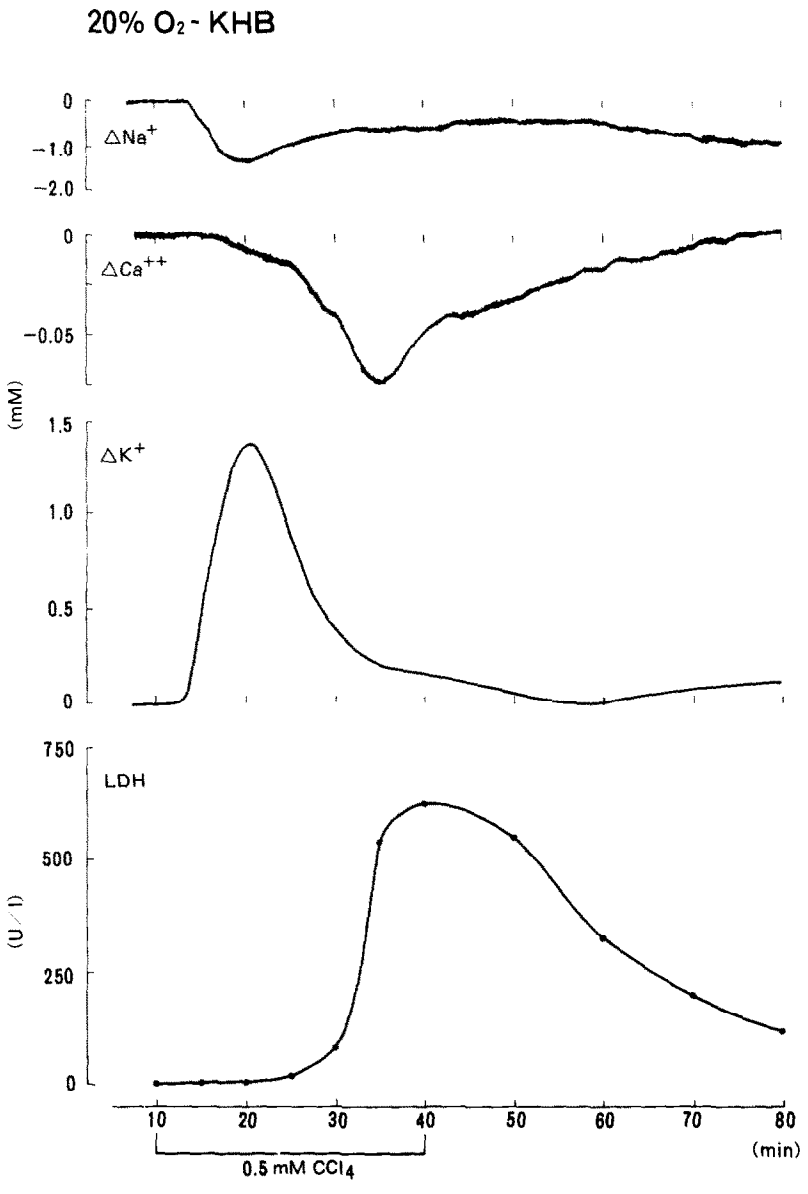

Fig. 5. Example of simultaneous recordings of the effluent ionic concentrations and LDH leakage following infusion of CCl_4 in the isolated, PB-pretreated rat liver, perfused with 20% O_2-saturated KHB.

perfusate following an instantaneous increase in oxygen uptake. This elevation was evident in PB-pretreated rat livers, but was quite low in normal rat livers (data not shown), and greatest after infusion of $CBrCl_3$ which has greater lipid peroxidative activity and hepatotoxicity than CCl_4 [34]. These indicate that the perfusate TBA-RS level reflects the extent of cellular lipid peroxidation. TBA-RS release by CCl_4 occurred under two different conditions: the absence of LDH leakage, such as observed under high oxygen supply (95% O_2) or in the early phase of low oxygen supply (20% O_2), and coupled with the LDH leakage such as observed in the later phase under low oxygen supply. The latter case indicates that in the hepatocytes, there accumulated considerable amounts of TBA-RS which may be metabolized further unless cell necrosis occurs, and also that lipid peroxidation occurred in prenecrotic hepatocytes.

The extent of TBA-RS release following CCl_4 infusion did not greatly differ between 20% O_2- and 95% O_2-KHB, despite a considerable difference in total oxygen uptake and pericentral oxygen concentration. This indicates that cellular lipid peroxidation occurs to a similar extent under both experimental conditions. Nevertheless, necrosis occurred only under low oxygen supply. This may be due to an increased cellular concentration of reductive metabolites of CCl_4 ($\cdot CCl_3$) and their covalent binding to cellular constituents, resulting

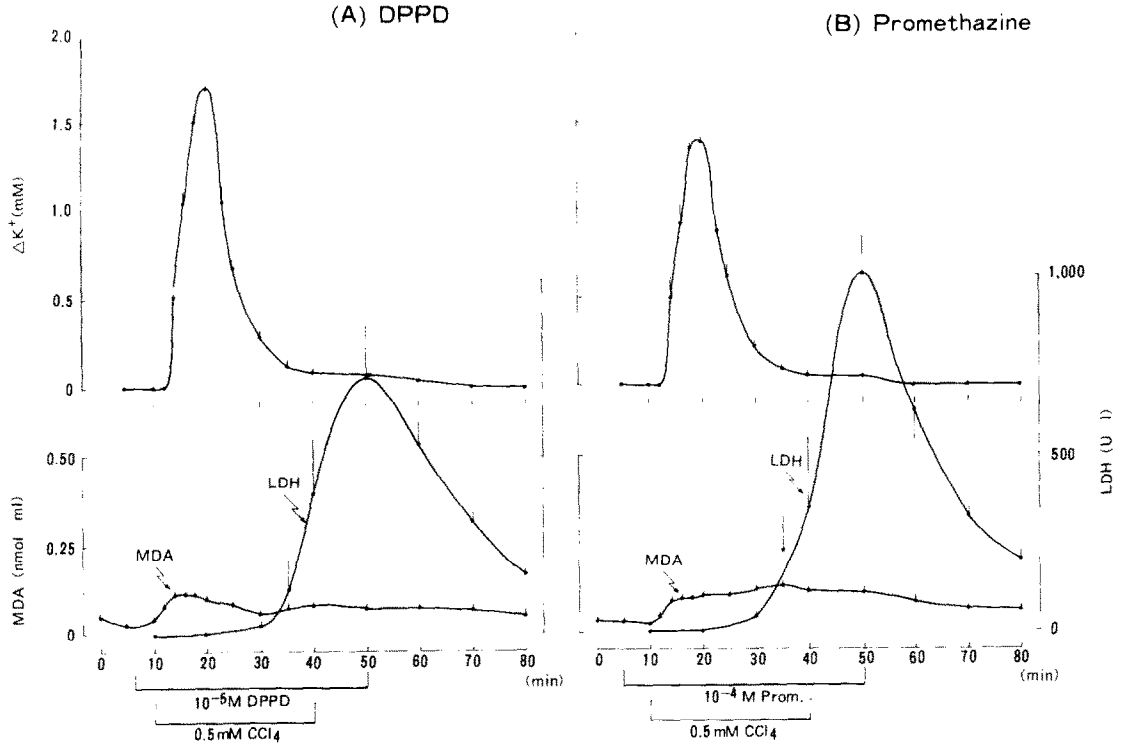

Fig. 6. Effects of DPPD (A) and promethazine (B) on the CCl_4-induced leakage of K^+, TBA-RS and LDH in the isolated, PB-pretreated rat liver, perfused with 20% O_2-saturated KHB. Values are means ± SE (N = 5).

from greater production of $\cdot CCl_3$ and a decreased conversion to $\cdot OOCCl_3$ under low oxygen tension. In support of this, hypoxia is reported to increase covalent binding of CCl_4 metabolites to cellular macromolecules *in vivo* [4]; metabolism of CCl_4 in the perfused liver, as measured by carbon dioxide anion production, is much faster during perfusion with nitrogen-saturated rather than oxygen-saturated perfusate, accompanying a more rapid LDH release [5]; and low oxygen pressure also increases covalent binding of CCl_4 metabolites to microsomes *in vitro* [7]. However, the covalent binding in the perfused liver still remains to be examined.

Furthermore, in the present study, $CBrCl_3$ caused far greater release of TBA-RS than CCl_4 under high oxygen supply, but with no accompanying necrosis. DPPD and promethazine, lipid peroxidation inhibitors which do not affect drug metabolism [35], suppressed CCl_4-induced TBA-RS production, but did not ameliorate necrosis. Histologic examinations confirmed that necrosis always occurred in efferent lobular areas, where oxygen concentration is lower, in both antero- and retrograde perfusions.

Thus, low oxygen tension may be a critical factor for CCl_4-induced liver cell necrosis. In addition, the present results are in favor of the idea that covalent binding of active metabolites of CCl_4, rather than lipid peroxidation itself, may be more important as a triggering event in the necrosis, although auxiliary roles of lipid peroxidation cannot be ruled out. *In vivo*, lower oxygen tension as well as higher activity of the cytochrome P450 monooxygenase system in the pericentral area [25] may also contribute to the preferential necrosis in this area.

The exact relationship between oxygen concentration and lipid peroxidation is difficult to ascertain in the isolated liver because of the marked oxygen gradient across the liver lobule [36, 37], and is also complicated by the dual effects of oxygen in the CCl_4-induced lipid peroxidation. That is to say, oxygen is definitely required for lipid peroxidation, while it competitively inhibits bioactivation of CCl_4, thus suppressing lipid peroxidation. In our study, when 95% O_2-KHB was used as the perfusate, the influent and effluent oxygen concentrations were about 0.62 and 0.13 mM respectively. The effluent oxygen concentration further decreased to about 0.03 mM following CCl_4 infusion. With 20% O_2-KHB, the oxygen concentration gradient was from 0.2 to 0.02 ~ 0.04 mM. If the pO_2 value of 640 mm Hg (0.81 mM) reported for a similar perfusion medium [38] is used, the effluent oxygen concentration under 20% O_2 supply will have a pO_2 value of 16–32 mm Hg. This value is not far from the free oxygen concentration in the rat hepatic vein (30–35 mm Hg, 43–50 μM) [39].

De Groot *et al.* [6], using an oxystat system and hepatocyte suspension, demonstrated that loss of cell viability occurred only when the initial phase of lipid peroxidation proceeded at a significant rate preferentially under low pO_2 (3–35 mm Hg), which seems to agree with most of our observations in the

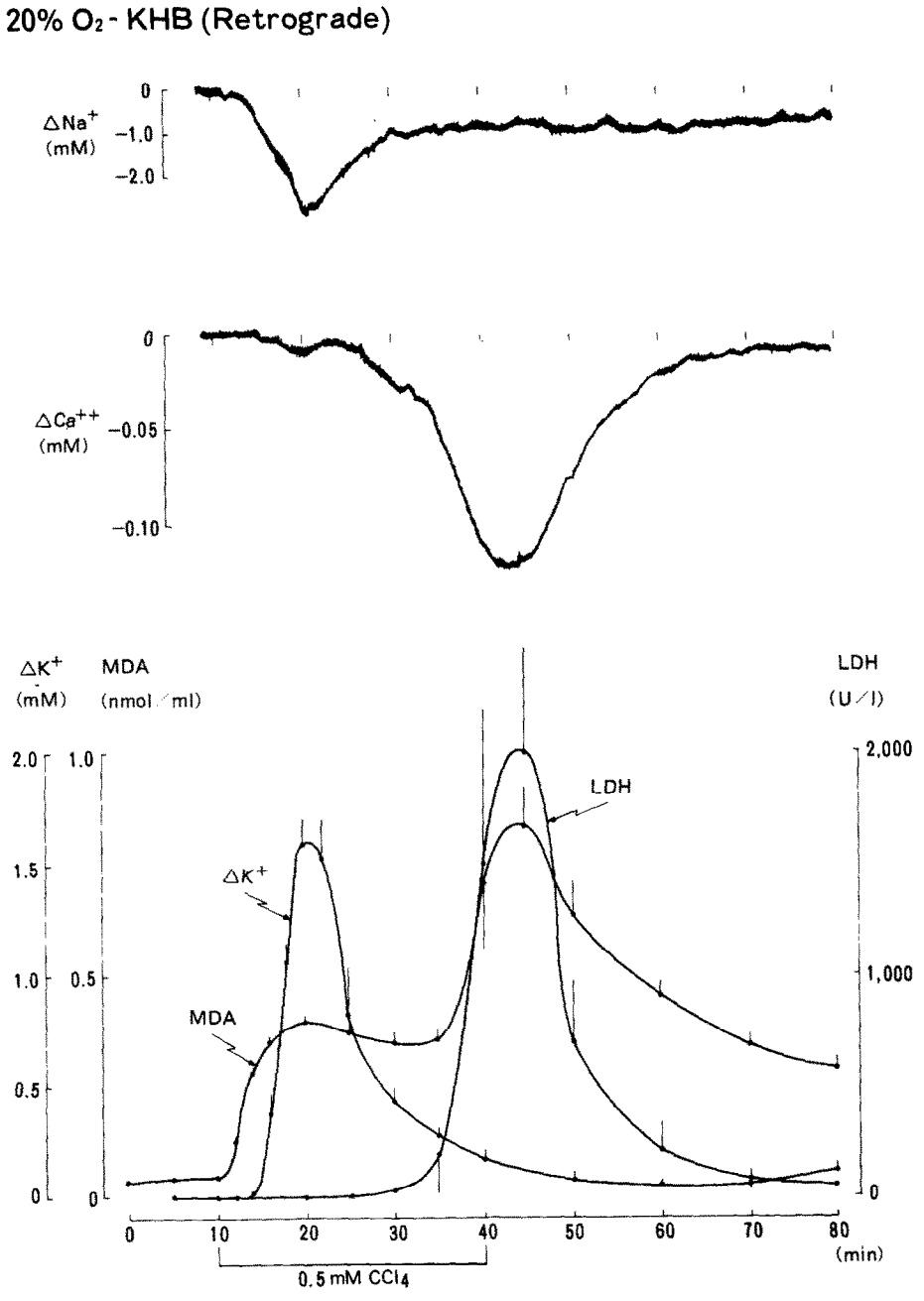

Fig. 7. Leakage of K^+, TBA-RS and LDH following infusion of CCl_4 in the isolated, PB-pretreated rat liver, perfused with 20% O_2-saturated KHB in a retrograde direction. Upper traces for Na^+ and Ca^{2+} were obtained from a typical experiment in which K^+ and LDH leakage had the same peak times as those shown in the bottom figure.

perfused liver. However, their conclusion that the initial phase of CCl_4-induced lipid peroxidation is the triggering event leading to ultimate cell death is different from ours, although it was noted that correlation between the initial phase of lipid peroxidation and cell injury is not complete, e.g. at a pO_2 of 3 mm Hg, loss of cell viability was enhanced significantly as compared to that at pO_2 of 7 mm Hg, whereas the lipid peroxidation rate showed the opposite behavior. In the perfused liver system, on the other hand, the reason why midzonal hepatocytes seemed to be more sensitive is unknown. Further studies may be necessary to lead to a final conclusion.

Calcium is now considered to mediate cell death by various toxic stimuli [40–42]. Among various hepatotoxic agents, CCl_4 and related halogenomethanes are rather unique in that they increase liver calcium more than twenty times that

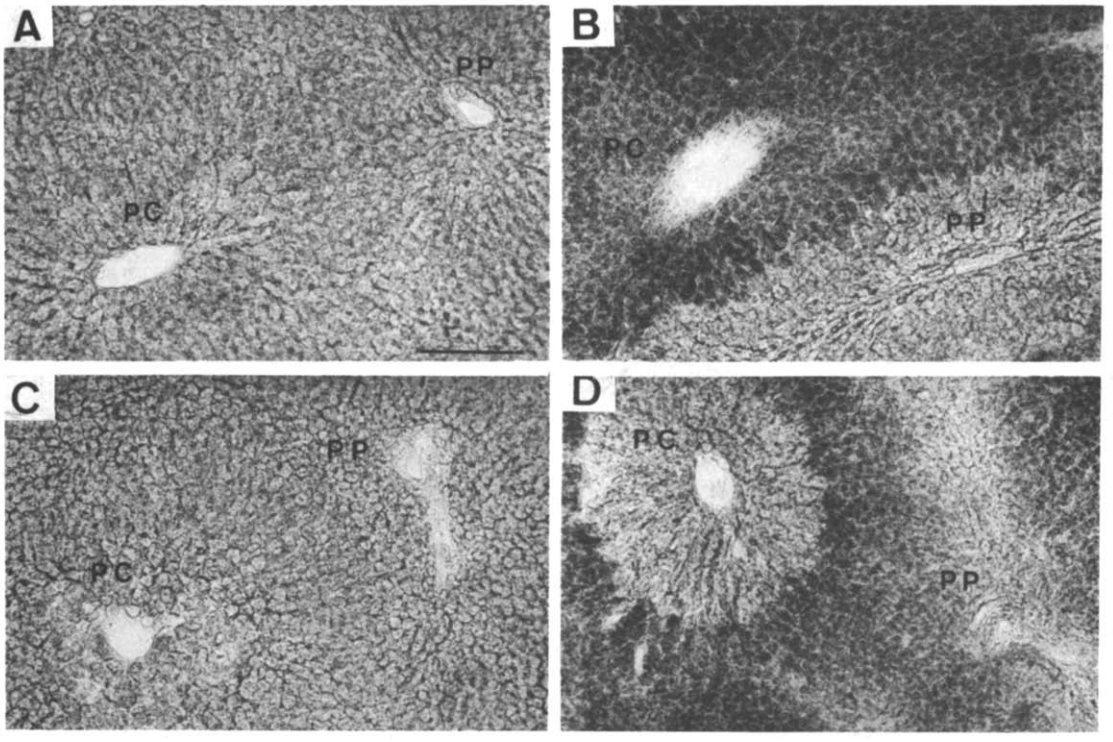

Fig. 8. Microscopic observations on the trypan blue uptake by hepatocytes following CCl_4 infusion in the isolated PB-pretreated rat liver. Liver was (A) perfused with 95% O_2-KHB and infused with CCl_4, 120 min; (B) perfused with 20% O_2-KHB and infused with CCl_4, 80 min; (C) perfused with 20% O_2-Ca^{2+}-free KHB and infused with CCl_4, 80 min; and (D) perfused with 20% O_2-KHB in a retrograde direction and infused with CCl_4, 80 min. PC and PP designate pericentral and periportal areas respectively. The scale shown in A denotes 0.2 mm (same magnification through A to D).

of control levels [9–11]. However, the requirement of extracellular Ca^{2+} for CCl_4-induced cell death is still controversial.

In the present experiments with isolated livers, perfused with 20% O_2-KHB, omission of Ca^{2+} from the medium markedly suppressed the LDH leakage following CCl_4 infusion. This cannot result from either suppression of lipid peroxidation or a decrease in CCl_4 metabolism, since the initial rise in the effluent TBA-RS level occurred to the same extent irrespective of the presence or absence of Ca^{2+}. Moreover, Casini and Farber [15] reported that extracellular Ca^{2+} concentration had no effect on the extent of covalent binding of CCl_4 metabolites and also on the extent of lipid peroxidation in cultured hepatocytes. Thus, our observations support the hypothesis that extracellular Ca^{2+} is required in the process of CCl_4-induced hepatocellular necrosis.

An increase in intracellular free Ca^{2+}, which has been demonstrated in isolated hepatocytes [20–22], may be caused by intrusion of extracellular Ca^{2+} as shown in the present study as well as inhibition of the intracellular Ca^{2+} uptake mechanism such as by endoplasmic reticulum [12, 20]. Recently, an increase in cytosolic Ca^{2+} concentration has been proposed to mediate cell death through various processes involving perturbation of cytoskeletal organization and uncontrolled activation of Ca^{2+}-stimulated catabolic enzymes such as phospholipase A_2, proteases and endonucleases [43].

K^+ leakage, like LDH leakage, is widely used as a parameter for toxic cell death in cultured or isolated cells [44]. K^+ leakage in the perfused liver is also reported to occur as a direct effect of high concentrations of CCl_4; however, correlation with liver necrosis has not been assessed [45]. In our study, a marked K^+ leakage was observed soon after CCl_4 infusion under low oxygen supply, which always preceded the development of necrosis. The total K^+ leakage amounted to about 60–70 μmol/g liver or more than half of the intracellular K^+ (115 mEq/L of cell fluid). This estimation is roughly in agreement with apparent necrotic areas of the liver lobules examined by trypan blue uptake, indicating that K^+ leaked from pre-necrotic hepatocytes. Coupled with this K^+ leakage, nearly equimolar amounts of Na^+ were taken up. Ca^{2+} uptake occurred in a biphasic manner, i.e. the initial slight uptake was coupled with K^+ leakage and the succeeding greater uptake occurred concurrently with the initiation of LDH leakage. These observations suggest that permeability of the hepatocyte plasma membranes may change in an early stage of CCl_4-induced cell death. This membrane permeability change itself may be initiated by the presence of extracellular Ca^{2+}, since the K^+ leakage was greatly suppressed in the absence

of Ca^{2+}. Associated with the membrane permeability change, there may evolve a marked change in intracellular ionic concentrations involving intrusion of Ca^{2+}, which may then trigger dynamic morphologic changes in plasma membranes, i.e. formation of membrane blebs and their splitting off, resulting in LDH leakage, as demonstrated by Lemasters *et al.* [46].

Plasma membranes have long been proposed as a critical site for CCl_4 liver injury [40], and it has been reported that marker enzyme activities of liver plasma membranes, such as 5′-nucleotidase, Mg^{2+}- and Na^+,K^+-ATPase [47, 48] and ATP-dependent Ca^{2+} uptake by plasma membrane vesicles [14], decrease soon after administration of CCl_4 *in vivo*. This study supports the significance of early plasma membrane injury in CCl_4-induced liver necrosis.

In conclusion, low oxygen tension is critical in the development of CCl_4-induced hepatocellular necrosis, in which covalent binding rather than lipid peroxidation may have a significant role in cooperation with Ca^{2+}. Irreversible changes in plasma membrane permeability may be one of the early and fatal signs of cell death.

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