STUDIES ON THE FUNCTION OF CELL MEMBRANE
10TH REPORT: EFFECTS OF CCl₄ ON THE MARKER ENZYME
ACTIVITIES AND FINE STRUCTURES OF RAT LIVER
PLASMA MEMBRANES AND MICROSOMES IN VITRO

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Abstract—Direct action of CCl₄ on rat liver plasma membranes and microsomes was investigated biochemically and morphologically in vitro. Plasma membranes or microsomes were pre-incubated with CCl₄, then subjected to enzyme assays and electron microscopic observations. In plasma membranes, Mg²⁺-ATPase lost its activity remarkably corresponding to the amount of CCl₄, whereas (Na⁺-K⁺)-ATPase remained unaffected. 5'-nucleotidase and leucine aminopeptidase activities decreased to some extent but only with a large amount of CCl₄. NADH-cytochrome c reductase activity also decreased, but not NADH-ferricyanide dehydrogenase activity. Some alterations of unit membrane structure and a complete disappearance of hexagonal patterns were observed electron microscopically in CCl₄-pretreated plasma membranes. In microsomes, a marked loss of glucose-6-phosphatase activity was detected. NADH- and NADPH-cytochrome c reductase activities also decreased to some extent, whereas NADH-ferricyanide dehydrogenase activity remained unchanged. Some of these results show similar tendencies to those observed with the in vivo administration of CCl₄. Thus, alterations of plasma membranes in vivo may indeed be partly due to a direct action of CCl₄.

In previous papers, we reported that NADH-cytochrome c reductase activity of liver plasma membranes increases markedly in an early period after the administration of CCl₄ to rats while no significant changes are observed in some enzyme activities of mitochondrial or microsomal electron transport systems, and that, contrary to the reductase, marker enzyme activities of membranes such as ATPase and 5'-nucleotidase activities decrease considerably (1, 2). Moreover, electron microscopic observations revealed ultrastructural changes of plasma membranes at this stage as evidenced by the disappearance of elementary particles and a hexagonal subunit pattern (3). Thus, we proposed that plasma membranes are the initial or very early site for liver injury provoked by CCl₄. Furthermore, it has been suggested from enzymatic studies that the elevation of NADH-cytochrome c reductase activity in membranes results not from the contamination of other subcellular fractions to the membrane fraction (2), but from the migration of microsomal cytochrome b₅ to plasma membranes (4, 5), thus, manifesting a simultaneous disturbance between plasma membranes and endoplasmic reticulum. These biochemical and morphological changes in plasma membranes observed in the early stage of CCl₄-induced liver lesion may be partly due to a direct action of CCl₄.
Taking such into consideration, we studied the in vitro effects of CCl₄ on plasma membranes and microsomes in terms of marker enzyme activities and electron microscopic observations.

MATERIALS AND METHODS

Female rats of Sprague-Dawley strain, weighing 200–250 g, were used throughout. Liver plasma membranes and microsomes were prepared according to the procedure described in our previous papers (1, 4). Fresh plasma membrane fractions were suspended in 150 mM KCl-25 mM Tris-HCl buffer (pH 7.5) with high protein concentrations, kept in an ice bath and used within 12 hr after the isolation. Microsomes were stored in the state of pellets at -20°C, and used after suspension in the same buffer solution.

Each fraction was treated with CCl₄ as shown in Fig. 1. Each 2 ml of the membrane (2 mg protein/ml) or microsomal (5 mg protein/ml) suspension was placed in a test tube of same size cooled in an ice bath, then CCl₄ was added in a total amount of 1–50 μl by using a micropipette. As soon as CCl₄ was added, the test tube was tightly capped with a rubber stopper to prevent the loss by evaporation and mixed vigorously using a mechanical mixer for about 15 sec. In the buffer solution not containing the cellular fractions, CCl₄ was well dispersed into fine particles for a short time and a fairly rapid precipitation occurred when 50 μl of CCl₄ was added. In the presence of the either fraction, however, CCl₄ precipitate was not evident up to 10 μl, although with 50 μl of CCl₄ turbid CCl₄ layer and precipitates became evident during an early incubation period. Such is attributed to both extraction of lipids and denaturation of proteins of the fractions. Thus, because of the insolubility in an aqueous solution and the high volatility of CCl₄, actual concentration of CCl₄ in the incubation system is difficult to assess, or rather because of the high solubility of CCl₄ in lipid it would be more important to determine how much of CCl₄ dissolved into.
lipids and hydrophobic sites of proteins in a membrane structure.

The mixtures of CCl₄ and cellular fractions thus prepared were incubated with shaking at 37°C or in an ice bath (at 0°C). At 20, 60 and 180 min, an aliquot of the mixture was removed by using a 1 ml syringe after which enzyme assays or electron microscopic observations in an open system were carried out.

The following enzyme activities were measured according to the methods given in the references: Mg²⁺- and (Na⁺-K⁺)-ATPase (6), 5'-nucleotidase (6), leucine aminopeptidase (7), glucose-6-phosphatase (8), NADH-cytochrome c reductase (9), NADH-frericyanide dehydrogenase (10) and NADPH-cytochrome c reductase (11). Protein was determined by the method of Lowry et al. (12). Each point in the graphs represents a mean value of 2–3 experiments.

Procedures for electron microscopic observations have been described elsewhere (3).

RESULTS

Effects of CCl₄ on the enzyme activities of liver plasma membranes

5'-nucleotidase, leucine aminopeptidase and ATPase were chosen as marker enzymes of plasma membranes. Activities of the former two enzymes were little affected when incubated at 37°C with small amounts of CCl₄, although 50 μl of CCl₄ caused some loss of activity.

On the other hand, Mg²⁺-ATPase was fairly sensitive to CCl₄ as compared with the above two enzymes. The activity decreased corresponding to the amounts of CCl₄ added, reaching 10°, that of the control in the presence of 50 μl of CCl₄ (Fig. 2). A rapid loss

![Fig. 2. Effect of CCl₄ on ATPase activity of liver plasma membranes in vitro (at 37°C).](image-url)
TABLE 1. Mg$^{++}$- and (Na$^{+}$-K$^{+}$)-activated ATPase activities of liver plasma membranes incubated with CCl$_4$ at 0°C.

| Incubation | CCl$_4$ (µl/2 ml) | Mg$^{++}$- | (Na$^{+}$-K$^{+}$)-activated |
|------------|-------------------|------------|-----------------------------|
| (20 min)   |                   |            |                             |
| 0          | 49.0              | 12.5       |
| 1          | 49.3              | 15.1       |
| 2          | 44.3              | 14.1       |
| 5          | 31.9              | 15.6       |
| 10         | 21.5              | 16.6       |
| 50         | 13.2              | 10.9       |
| (180 min)  |                   |            |                             |
| 0          | 49.3              | 12.8       |
| 1          | 49.6              | 15.3       |
| 2          | 46.4              | 12.3       |
| 5          | 35.0              | 14.6       |
| 10         | 25.5              | 17.1       |
| 50         | 14.2              | 11.3       |

of activity was observed within 20 min. Similar results were obtained with Mg$^{++}$-ATPase when membranes were incubated with CCl$_4$ at 0°C, though the degree of the inhibition was less. In this case, (Na$^{+}$-K$^{+}$)-ATPase activity was assayed at the same time. As shown in Table I, contrary to the remarkable loss of Mg$^{++}$-ATPase activity, (Na$^{+}$-K$^{+}$)-ATPase activity remained virtually unaffected.

According to a preliminary report by Dorling and Le Page (13), both ATPase activities were inhibited in the presence of CCl$_4$ and recovered partially when CCl$_4$ was evacuated. In their experimental conditions, CCl$_4$ was diffused to the reaction mixture in a Warburg vessel. We also tried a similar experiment by transferring 2 ml of the CCl$_4$-pretreated membrane suspension to a small Meyer flask and evacuation was carried out for 10 min. No recovery in the activity, however, was observed, rather under our experimental conditions, Mg$^{++}$-ATPase activity was irreversibly inhibited.

NADH-cytochrome c reductase and NADH-ferricyanide dehydrogenase, the activities of which increased markedly in liver plasma membrane fractions after the administration of CCl$_4$ to rats (1), were also examined in vitro. These enzyme activities were rapidly lost during the incubation at 37°C even without CCl$_4$, therefore, membranes were incubated with CCl$_4$ at

Fig. 3. Effect of CCl$_4$ on NADH-cytochrome c reductase activity of liver plasma membranes in vitro (at 0°C).
0°C. CCl₄ caused a decrease in the reductase activity (Fig. 3), while there was no effect on the dehydrogenase activity.

Effects of CCl₄ on microsomal marker enzyme activities

Glucose-6-phosphatase, NADH- and NADPH-cytochrome c reductase and NADH-ferricyanide dehydrogenase were selected as microsomal marker enzymes. These microsomal enzymes were very labile to the incubation at 37°C, especially the latter three enzymes, in which cases microsomes were incubated with CCl₄ at 0°C.

In the presence of CCl₄, glucose-6-phosphatase activity was lost progressively with less quantities of CCl₄ and very rapidly with 10–50 μl of CCl₄ at 37°C (Fig. 4). The loss of the activity by CCl₄ was less when incubated at 0°C. Unlike the case of plasma membrane Mg²⁺-ATPase, the decrease of microsomal glucose-6-phosphatase activity could be partially recovered by evacuation or by the addition of lecithin. As for the electron transport enzymes, both NADH- and NADPH-cytochrome c reductase activities decreased only with 10–50 μl CCl₄, but not so remarkable as in the case of glucose-6-phosphatase activity. NADH-ferricyanide dehydrogenase activity, however, was not changed as in the case of plasma membranes.

Electron microscopic observations on plasma membranes treated with CCl₄

Fig. 5 shows a positively stained fresh membrane fraction in which unit membrane

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**Fig. 5.** Electron micrograph of the liver plasma membrane fraction isolated from normal rats. Positively stained.

a) Low magnification (x 30000)
b) High magnification of pentalayered tight junction (x 80000)

**Fig. 6.** Liver plasma membrane fraction 20 min after treatment with 10 μl of CCl₄ at 0°C. Positively stained.

a) Low magnification (x 30000)
b) High magnification of pentalayered tight junction (x 80000)
structures (approx. 90 Å in width) and pentalayered tight junctions (approx. 140 Å in width) are clearly observed as reported by Neville (14) and Emmelot et al. (15). When plasma membranes were incubated with 10 µl of CCl₄ at 0°C or at 37°C, these structures became obscure and the adhesion of small particles having high electron density was observed (Fig. 6), while no structural changes were detected in the absence of CCl₄ during a 180 min incubation at both temperatures.

In Fig. 7, a higher magnification of a negatively stained membrane sheet is shown. When membranes were negatively stained after treatment with deoxycholate, a hexagonal pattern consisting of unit structures having a diameter of approx. 50 Å appeared as described by Benedetti and Emmelot (16, 17) (Fig. 8). The hexagonal pattern was well preserved after the incubation of membranes either for 180 min at 0°C or for 20 min at 37°C. Prolonged incubation at 37°C for more than 60 min resulted in the disappearance or deformation of this structural profile. Therefore, in order to examine the effect of CCl₄, plasma membranes were incubated at 0°C. A large irregularly shaped hole-like appearance was visible in the membrane sheet and the hexagonal pattern disappeared almost or completely after 20 min of incubation with 5-10 µl of CCl₄, as shown in Figs. 9 and 10. The degree of
deformation became demarcated as the incubation time was prolonged. These ultrastructural alterations of plasma membranes in the presence of CCl₄ are quite similar to those observed in the in vivo experiments (3).

DISCUSSION

Although an exact mechanism involved in the CCl₄-induced liver damage has yet to be established, it seems quite certain that CCl₄ acts directly on the liver (18-20). It has been suggested that an attack point of CCl₄ is the endoplasmic reticulum of liver cells (20, 21). On the other hand, we have recently demonstrated that surface membranes of liver cells would be the first place where circulating CCl₄ exhibits its effects (1-3).

Direct actions of CCl₄ on these subcellular fractions in vitro were investigated in an attempt to compare them with the biochemical and morphological changes observed in vivo.

The in vitro effects of CCl₄ on certain enzyme activities of liver plasma membranes

![Comparison of the effects of CCl₄ on various enzyme activities of liver plasma membranes in vitro at 37°C (a, b) or at 0°C (c-f). Incubation time: 20 min.](image)
and microsomes are summarized in Figs. 11 and 12. It is obvious that the activity of each enzyme is affected differently.

In plasma membranes, Mg\textsuperscript{2+}-ATPase, but not (Na\textsuperscript{+}-K\textsuperscript{+})-ATPase, was affected considerably by CCl\textsubscript{4}. These enzymes are markers of plasma membranes, and are highly dependent on lipids since both of their activities are lost by lipid extraction or phospholipase treatment of membranes (6). This indicates that CCl\textsubscript{4} disturbs the lipid structure of membranes, causing a loss of Mg\textsuperscript{2+}-ATPase activity. It is interesting, however, that (Na\textsuperscript{+}-K\textsuperscript{+})-ATPase activity did not decrease when Mg\textsuperscript{2+}-ATPase activity was to a great extent lost.

5\textsuperscript{'}-nucleotidase and leucine aminopeptidase, the other marker enzymes of plasma membranes located at the outer surface of plasma membranes, were much less affected than Mg\textsuperscript{2+}-ATPase. The activities of these enzymes are reportedly not dependent on lipids (6, 22).

NADH-cytochrome c reductase activity was inhibited as the amount of CCl\textsubscript{4} added was increased, but NADH-ferricyanide dehydrogenase activity remained unchanged. NADH-cytochrome c reductase system of plasma membranes has similar characteristics to that of microsomes and contains cytochrome b\textsubscript{5} as a co-factor (4), which, in microsomes, was proven to be associated with lipid structure through the hydrophobic portion of the protein (23). On the other hand, the dehydrogenase—a flavoprotein portion of the reductase—has no requirement for structural lipids as the enzyme is extractable from microsomes by phospholipase treatment (24). Accordingly, cytochrome b\textsubscript{5} bound to plasma membranes may become loose or detached by the treatment of membranes with CCl\textsubscript{4}, thus causing loss of the reductase activity.
In microsomes, as reported by other workers (25, 26), glucose-6-phosphatase activity decreased remarkably by CCl\(_4\) treatment. This enzyme is one of the most lipid-dependent ones (27). A similar discussion to that cited above may also be adapted to microsomal reductase and dehydrogenase.

These findings indicate that the greater the lipid dependence of the enzyme, the more susceptible is the enzyme to CCl\(_4\) treatment, with the exception of (Na\(^+-K^+\))-ATPase.

Electron microscopic observations also showed alteration of a unit membrane structure or the disappearance of a hexagonal subunit pattern in the presence of CCl\(_4\). Such may be correlated with the loss of enzymatic activities.

As CCl\(_4\) is highly soluble in lipids, a small amount of CCl\(_4\) may dissolve into lipid components of membraneous structures and a large amount may even extract membrane lipids, thus producing biochemical as well as morphological changes in both plasma and microsomal membranes. Furthermore, preliminary experiments on the recovery of enzyme activities by evacuation or by the addition of lecithin suggest that plasma membranes are irreversibly damaged by CCl\(_4\) whereas microsomal damages are reversible.

Another objective of this experiment was to see if any correlation exists between in vivo and in vitro effects of CCl\(_4\) on liver plasma membranes.

From the following points: (1) the decrease of Mg\(^{++}\)-ATPase but not for (Na\(^+-K^+\)) -ATPase activity, observed after in vivo administration of CCl\(_4\) was also detected in vitro, (2) ultrastructural changes of plasma membranes in vivo were comparable to those observed in vitro, these in vivo actions of CCl\(_4\) on plasma membranes may perhaps be due to a direct actions of CCl\(_4\) since a metabolic breakdown of CCl\(_4\) is not expected to have occurred in our experimental conditions.

5'-nucleotidase activity, in contrast with that of Mg\(^{++}\)-ATPase, was little affected by CCl\(_4\) in vitro, although both activities decreased after CCl\(_4\) administration, which suggests that different mechanisms are involved. The possibility that membrane bound 5'-nucleotidase is released into the blood stream by disturbance or destruction of the plasma membrane, as the enzyme is solubilized by treatment with detergents in vitro (22), cannot be overlooked.

NADH-cytochrome c reductase in the membranes, which increases markedly in vivo, decreased in vitro. This apparently quite opposite effect leads to the consideration that CCl\(_4\) would first attack plasma membranes, causing a release of cytochrome b\(_5\), after which cytochrome b\(_5\) simultaneously released from endoplasmic reticulum binds to the plasma membranes in a greater amount than originally existed bringing about a rise in the reductase activity. Ascertainment of this point is under study.

On the other hand, microsomal glucose-6-phosphatase appears to be more sensitive to CCl\(_4\) than plasma membrane Mg\(^{++}\)-ATPase in vitro, although in the in vivo experiments, the decrease of the former enzyme activity was relatively small as compared with that of the latter in an early stage of the liver damage. In a later stage or with a larger dose of CCl\(_4\), however, a considerable decrease of the activity is reported (25, 28, 29). Accordingly, the decrease of microsomal glucose-6-phosphatase activity in vivo may be produced
by CCl₄ per se as well as its more toxic metabolite as suggested by other workers (20, 21, 30).

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