EFFECT OF CARBON TETRACHLORIDE UPON ARYLESTERASES IN MICE

NORIMITSU TAKAHASHI AND MASAO NAKAZAWA
Division of Pharmacology, Taisho Pharmaceutical Co., Ltd., Toshima-ku, Tokyo

TADASHI WATANABE
Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo

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Early works by Augustinsson (1–4) demonstrated that three types of esterases exist in vertebrate plasma in regard to substrate specificity and susceptibility to selective inhibitors, and he classified them into arylesterases [EC 3.1.1.2], aliesterases (carboxylesterases) [EC 3.1.1.1] and cholinesterases [EC 3.1.1.8]. However, physiological and clinical significance that the altered activities of these esterases imply is still obscure. Recently, Takahashi et al. (5) suggested that decrease in the activity of phenyl acetate esterase (corresponding to arylesterases, according to the Augustinsson’s classification) was correlated with the degree of liver damage and that, clinically, the activity of the enzyme in serum decreased greatly in the case of liver cirrhosis. In the present paper, changes in the total activity and the electrophoretic pattern of arylesterases were studied in CCl₄-induced mouse liver injury using β-naphthyl acetate as substrate.

METHODS

Experiment I: Effect of CCl₄ administration upon electrophoretic pattern of arylesterases in liver 12,000 × g supernatant and serum

Male inbred mice of the NC strain weighing 20 to 24 g were divided into 7 groups of 5 animals each, and treated with 1 ml/kg of CCl₄ intraperitoneally except the control group. They were sacrificed at 1/4, 3, 6, 24, 48 and 72 hours after CCl₄ injection by exsanguination from the femoral artery. The liver was immediately removed, rinsed with ice-cold physiological saline and homogenized with an equal amount of 0.05 M phosphate buffer, pH 7.4, by a glass homogenizer with a teflon pestle under refrigeration. The subsequent procedures were performed at 0°C. The homogenate was centrifuged for 45 minutes at 12,000 × g and the supernatant was separated. The blood collected was also centrifuged for 15 minutes at 3,000 r.p.m. to obtain serum.

Thin-layer agar gel electrophoresis described by Ogita (6–8) was performed for 2 hours with the constant current adjusted to 1 mA/cm to separate arylesterases in the supernatant of liver homogenate and serum. After electrophoresis, the glass plate covered with agar gel film was immersed into phosphate buffer, pH 6.8, containing β-naphthyl acetate as substrate and naphthyl diazo blue B and kept at 37°C for 20 minutes. The
Experiment II: Effect of CCl₄ administration upon electrophoretic pattern and total activity of arylesterases in liver subcellular fractions and serum

The animals of the same strain described previously were divided into 5 groups of 9 animals each and similarly treated with 1 ml/kg of CCl₄ intraperitoneally. The animals were killed at 6, 12, 24 and 48 hours after administration, and the liver and blood were obtained as outlined previously. The liver was homogenized with 9 volumes of 0.25 M sucrose solution, and the debris and nuclei were removed by centrifugation for 10 minutes at 900 x g. The mitochondrial fraction was separated by centrifugation for 10 minutes at 8,000 x g. The unsedimentable fraction was centrifuged again for 1 hour at 105,000 x g (Hitachi Model 55PA Ultracentrifuge), giving the supernatant and microsomal fractions. The mitochondrial and microsomal fractions were dissolved in an appropriate volume of 0.05 M phosphate buffer, pH 7.4, containing 0.1 % DOC. The blood was also centrifuged for 15 minutes at 3,000 r.p.m. to obtain serum in pools of 3 animals in each group.

Electrophoresis was carried out as described previously.

The outline of the determination of total arylesterase activity was as follows:

Serum and each fraction of liver homogenate diluted appropriately were incubated for 30 minutes at 37°C with 0.05 M phosphate buffer, pH 7.4, containing β-naphthyl acetate as substrate. Reaction was terminated by adding TCA solution, and naphthanyl diazo blue B solution was added, resulting in formation of insoluble complex with β-naphthol released by the action of arylesterases. The resultant complex was extracted by ethyl acetate, and the amount dissolved in it was determined spectrophotometrically at 550 mμ.

Alkaline phosphatase [EC 3.1.3.1] activity was determined by Kind and King method (9), glutamic-oxalacetic and glutamic-pyruvic transaminase [EC 2.6.1.1 and EC 2.6.1.2, respectively] activities were determined by Reitman and Frankel method (10). Protein in each fraction was measured by the method of Lowry et al. (11).

Experiment III: In vitro effect of CCI₄ upon electrophoretic pattern of hepatic arylesterases

Liver of healthy mice of the NC strain was homogenized with an equal amount of 0.05 M phosphate buffer, pH 7.4, in pools of 20 animals. One ml of homogenate thus obtained was incubated for 0, 10, 20, 40, 80, 160 and 320 minutes at 37°C together with 0.2 ml of 20% non-ionic surfactant (HCO 50*) solution containing CCl₄ at the concentration of 10, 5, 2.5, 1.25 and 0%. Each homogenate incubated for a definite time was centrifuged for 45 minutes at 12,000 x g and the supernatant was submitted to electrophoretic separation of arylesterases as described previously. On the other hand, 5 volumes of 0.25 M sucrose solution was added to liver homogenate which had been incubated for 320 minutes with solution containing 10% CCl₄ and the mixture was fractionated by ultracentrifugal procedure as outlined previously. The mitochondrial and microsomal fractions were treated similarly with 0.1% DOC and also submitted to electrophoresis.

* Manufactured by Nikko Chemicals Co., Ltd.
Experiment I: Effect of CCl₄ administration upon electrophoretic pattern of arylesterases in liver 12,000 × g supernatant and serum

Time course of changes in the electrophoretic pattern of serum arylesterases in mice sacrificed following single intraperitoneal administration of CCl₄ is presented in Fig. 1. Bands a and h' exist originally in the serum of normal mice as seen in the control, when α- or β-naphthyl acetate is used as substrate. After 15 minutes following injection, no apparent change in the pattern was observed. After 3 hours, however, band b appeared clearly, and weak bands f, i and k were occasionally seen. After 6 hours, bands b, c, f, i, k and l were recognized clearly. After 24 hours, band b disappeared, and instead definite bands c and d were seen. Bands f, i, j, k and l were also recognized. After 48 hours, bands c and d were reduced in their intensities, but bands f, i, j, k and l were unchanged. After 72 hours, bands

Fig. 1. Effect of CCl₄ administration upon electrophoretic pattern of serum arylesterases.
Mice treated with 1 ml/kg of CCl₄ intraperitoneally were sacrificed at indicated intervals following administration. Blood was collected and serum was obtained. Serum arylesterases were separated electrophoretically as described by Ogita (6-8) using β-naphthyl acetate as substrate.
b, c and d disappeared almost completely, but in some cases weak bands f, i, j, k and l remained.

The relationship between the interval after the treatment and electrophoretic pattern of hepatic arylesterases in the 12,000 × g supernatant following CCl₄ administration is shown in Fig. 2. After 1/4, 3 and 6 hours following injection, no distinct change in hepatic arylesterase patterns was recognized when compared with control, though it was uncertain whether the total activity was altered or not. After 24 hours, however, bands c, d, e, f, i, j and k became thicker and conversely, bands g and h became thinner. After 48 and 72 hours, the pattern itself was almost unaltered though the activity in each band, especially bands c, d, e, f, i, j and k, decreased.

**Experiment II: Effect of CO₂ administration upon electrophoretic pattern and total activity of aryl-esterases in liver subcellular fractions and serum**

The mobilization of hepatic arylolesterases from the microsomal fraction was studied...
by examining the electrophoretic pattern of subcellular fractions separated from liver homogenate and serum. Fig. 3 shows arylesterase patterns in serum and liver subcellular fractions of mice sacrificed after 24 hours following CCl₄ administration. In the control, the pattern in the liver 105,000 × g supernatant was not clear because it was diluted about 5-fold during fractionation as compared with the 12,000 × g supernatant in Fig. 2. On the other hand, a distinct pattern was recognized in the control microsomal fraction treated with DOC. The overall pattern in the supernatant of CCl₄-treated animals was more definite and stronger than that of the control, and had a striking similarity to the pattern in the control microsomal fraction except bands b, c and d, while the pattern in CCl₄-treated microsomal fraction was obscure and weak. In the serum of the CCl₄-treated animals, bands e, d, f, i, j, k and l were recognized in addition to those in the serum of the control as

![Electrophoretic pattern of arylesterases](image)

**Fig. 3.** Effect of CCl₄ administration upon electrophoretic pattern of arylesterases in liver subcellular fractions and serum.

Mice treated with 1 ml/kg of CCl₄ were sacrificed 24 hours after administration. Liver was removed, homogenized and separated electrophoretically as outlined in Fig. 1.

SER: Serum
SUP: Liver supernatant fraction
MIT: Liver mitochondrial fraction
MIC: Liver microsomal fraction
observed in Experiment 1, and the pattern reflected precisely that of the liver supernatant of the CC14-treated animals. The liver mitochondrial fractions of both the control and the CC14-treated animals showed no arylesterase activity.

The relationship between the interval after the treatment and the total arylesterase activities in serum, liver supernatant and microsomal fraction following CCl4 administration is shown in Fig. 4. In the microsomal fraction, stepwise decrease in enzyme activity was observed during the first 24 hours following CCl4 injection. From 24 to 48 hours, the enzyme activity was almost unaltered. In the liver 105,000 x g supernatant, the activity increased promptly within 24 hours following administration in contrast to that of the microsomal fraction. The maximum level in the activity was attained after 24 hours, followed by a decrease. In the serum, however, no change in the activity was found during the first 6 hours, followed by a decrease within 24 hours, and thereafter, the activity remained unchanged. No transient elevation in the serum arylesterase activity was confirmed, though the possibility that this enzyme in the liver leaked into the blood was suggested electrophoretically.

**Fig. 4.** Effect of CCl4 administration upon total activity of arylesterases in liver subcellular fractions and serum.

Mice treated with 1 ml/kg of CCl4 were sacrificed at indicated intervals following administration. Liver was removed, homogenized and separated into subcellular fractions. Serum was also obtained. Total activity of arylesterases in each fraction and serum was determined as described in Methods using 3-naphthyl acetate as substrate.

- x : Serum (right side scale)
- o : Liver supernatant fraction (left side scale)
- : Liver microsomal fraction (left side scale)

**Fig. 5.** Effect of CCl4 administration upon total activity of serum glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and alkaline phosphatase (ALP).

Mice treated with 1 ml/kg of CCl4 were sacrificed at indicated intervals following administration. Serum was obtained and total activity of GOT, GPT and ALP was determined as described in Methods.

- o - : GOT (left side scale)
- : GPT (left side scale)
- x : ALP (right side scale)
Time course of change in the activities of glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT) and alkaline phosphatase (ALP) in the serum are presented in Fig. 5. It is widely known that the activities of these enzymes in serum are sensitively elevated especially in acute liver injury. In the case of CCl₄-induced acute liver injury in mice, stepwise increase in the activity of GOT was seen for the first 24 hours, and thereafter it remained unchanged or slightly decreased. The activity of GPT was also elevated sharply and reached the maximum level after 24 hours, followed by a significant decrease. As to the activity of ALP, gradual elevation was recognized throughout the observation period of 48 hours.

**Experiment III: In vitro effect of CCl₄ upon electrophoretic pattern of hepatic arylesterases**

The relationship between the incubation period of liver homogenate with 10% CCl₄ and the pattern in the liver 12,000 x g supernatant is shown in Fig. 6. In the first 20
FIG. 7. In vitro effect of CCl₄ upon electrophoretic pattern of arylesterases in liver subcellular fractions.

One ml of mouse liver homogenate was incubated with 0.2 ml of 10% CCl₄ for 320 minutes and separated into subcellular fractions. Arylesterases in each fraction were separated electrophoretically as outlined in Fig. 1.

SUP: Liver supernatant fraction
MIT: Liver mitochondrial fraction
MIC: Liver microsomal fraction

minutes, the intensities of bands f, i, j and k elevated gradually with time and were maintained at the same level. Bands b and e (especially e) showed further increase in their intensities after 40 minutes of incubation. When incubated with 5, 2.5 or 1.25% CCl₄, longer incubation time was required for the increase of intensities of bands b, e, i, j and k. When liver homogenate was incubated with surfactant solution alone as the control, no alteration in the pattern was found during the incubation period of 320 minutes.

As seen in Fig. 7, the in vitro liberation of microsomal arylesterases to the supernatant fraction in the liver was also examined with subcellular fractions separated from liver homogenate incubated with CCl₄ for 320 minutes. A distinct pattern was recognized in the control microsomal fraction treated with DOC. The pattern in the supernatant of liver homogenate incubated with CCl₄ indicated considerably higher overall activity than that of the control and, moreover, had a close similarity to the pattern in the control microsomal fraction except band c. On the other hand, definite pattern in microsomal fraction of
homogenate incubated with CCl₄ was not shown, indicating weak overall activity of the enzyme contained therein. No influence of CCl₄ upon the arylesterase pattern of its mitochondrial fraction was observed.

DISCUSSION

In the present paper, the possibility that arylesterases in the liver were released into the blood was demonstrated in CCl₄-induced mouse hepatic injury, as shown in Fig. 1. The mechanism responsible for elevated serum enzyme activity was thought to be attributable to cell necrosis, but the possibility must be considered that permeability alteration may also be responsible for this finding (12). The diffusion of enzymes from apparently intact cells has been observed in the case of aldolase in rat muscle (13) and a number of glycolytic enzymes in ascites tumor cells (14). Henley et al. (15, 16) showed that considerable amount of GPT leaked into the suspending medium in the course of preparation of the morphologically intact rat liver cell suspension. Recent clinical observations (17) show that many enzymes appear in the serum of patients with hepatitis and these are very similar to those which leak out from dispersed liver cells (18). Moreover, some glucocorticoids have a suppressive action on leakage of some enzymes (18). Dinman et al. (19) reported that significant increase in serum enzyme activities of most non-mitochondrial, intracellular enzymes occurred immediately after exposure to CCl₄, in the absence of necrosis. At the same time, changes at cellular and subcellular levels suggesting altered permeability states were demonstrated by electron microscopic examination. As shown in Fig. 1, the liver arylesterases were liberated into the serum not at the same time, but those with higher mobilities were initially released, suggesting the possibility that arylesterases were also released into the serum by increase in the permeability of cell membrane, especially in the early stage of liver injury. However, Beneche and Simon (20) observed periportal, local necrosis in the liver after 1 hour following portal application of CCl₄. Although the molecular weight of the enzyme is of importance when it passes through the cell membrane (21), there exist many unsolved additional problems concerning the mechanisms of enzyme leakage brought about by alteration in the permeability of cell membrane. Besides, the stability of enzymes in the blood may also be questioned. For example, band b in Fig. 1 appeared within 6 hours and became reduced within 24 hours following administration.

As seen in Fig. 2, no distinct alteration in patterns in the liver 12,000 × g supernatant was observed during the first 6 hours following administration of CCl₄, but after 24 hours the intensities of bands c, d, e, f, i, j, and k increased, whereas those of bands g and h decreased. In the histological and histochemical study on the liver of rats treated with CCl₄ (22), enzymatic alteration is seen prior to the appearance of the histological change revealed by hematoxylin and eosin stain. The alteration produced by CCl₄ in the rough endoplasmic reticulum suggested that there might be a defect in protein synthesis (23). Electron micrograph showed a widespread dislocation of the ribonucleoprotein particles from the membranes of the rough endoplasmic reticulum, and simultaneous decrease in amino acid incorporation rate reflects depressed synthesis of protein by the liver (24). Since
these changes shown in Fig. 2, especially the elevation in enzyme activities in the supernatant at 24 hours, were hardly attributable to enzyme induction, arylesterase patterns in the liver subcellular fractions were also examined. As shown in Fig. 3, the pattern of microsomal fraction recognized in the control was almost completely disappeared after 24 hours following CCl₄ administration, and that the pattern in the supernatant reflected that of the control microsome except bands b, c and d. Thus, it appeared that the enzyme for band b was liberated rather earlier from the liver and became inactivated in the blood as mentioned previously. Enzymes for bands c and d seemed to be localized only in the supernatant and their elevation was observed in some cases. Though it seems possible that band b may be modified to bands c and d, further investigations will be needed to clarify this phenomenon and the disappearance of bands g and h in Fig. 2 after 24 hours as described above. As seen in Fig. 3, the pattern in the serum following administration completely agrees with that of the supernatant except bands a and h', which originally exist in the serum, indicating that arylesterases in the supernatant were transferred to the blood as they were.

Recent work (5) reported that the activity of phenyl acetate esterase (corresponding to arylesterases according to the Augustinsson's classification) in the serum, especially the highest peak in the activity found in the region of albumin or prealbumin, decreased in experimental liver damage in mouse or in cirrhosis in man. As shown in Fig. 4, in spite of the liberation of liver arylesterases into serum by the CCl₄ treatment, the total activity of arylesterases in the serum was not elevated but decreased. This may be explained by the fact that the most intensive band a in the normal serum zymogramm is reduced following CCl₄ administration and, in addition, by the fact that the enzymes liberated into the blood are generally unstable.

Cortisone and related corticosteroids are representative substances which stabilize the lysosomal or cell membrane; a number of compounds weaken the membrane, making it more permeable. CCl₄ is one of such compounds. The total activities of β-glucuronidase and acid phosphatase in the liver homogenate obtained by needle biopsy from subjects with acute hepatitis or active cirrhosis were generally reduced and the free activities increased resulting in elevation in the ratio of free/total activity possibly due to an increased fragility of lysosomal structure (25). Such reasoning seems to be applicable to the result in this experiment. As shown in Fig. 4, the ratio of free (in supernatant)/bound (in microsome) activity per protein was 0.4 in normal mice, whereas 4.3 in mice after 24 hours following CCl₄ administration. After 48 hours, the total activity was also lowered significantly.

As seen in Fig. 5, the activities of GOT, GPT and ALP, the enzymes generally thought as indicators of acute liver disease, were remarkably elevated following CCl₄ administration, whereas changes in total arylesterase activity was not so marked in this experimental condition. The study on qualitative alteration of arylesterases may be much more interesting than that of the quantitative one in acute liver injury.

When liver homogenate was incubated with CCl₄ in vitro, the arylesterase pattern in the 12,000 × g supernatant changed with time as presented in Fig. 6. This altered
pattern, especially that produced by incubation for 40 or 80 minutes, agrees with that obtained after 24 hours following CCl₄ administration except bands b, c and d as shown in Fig. 2. Increase in the intensity of band b may be partly due to its accumulation in the reaction mixture under the in vitro condition. The experimental result with subcellular fractions which is illustrated in Fig. 7 agrees completely with that of the in vivo experiment except the origin of band c.

In summary, the possibility was presented that arylesterases in the liver microsomal fraction was liberated into the supernatant, resulting in the leakage into the serum in CCl₄-induced mouse hepatic injury.

**SUMMARY**

The alteration in electrophoretic pattern and total activity of arylesterases in liver and serum of mice treated with CCl₄ was examined with the following results.

1. In the serum, changes in the electrophoretic pattern were detected as early as 3 hours following administration, and the newly appeared bands became maximum in their number after 24 hours.

2. In the liver 12,000 × g supernatant, no distinct change in the pattern was noted for the first 6 hours, but an altered pattern mainly characterized by elevation in their activities was recognized 24 hours after administration.

3. As a result of the study on subcellular fractions of liver, it was seen that the electrophoretic pattern and the total activity in microsomal fraction were transferred to the supernatant following administration resulting in leakage into the blood, but that the total arylesterase activity in the serum was lowered.

4. Similar changes observed in vivo in the liver following administration were reproduced in the in vitro experiment.

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