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# Hepatic Collagenolytic Activity in Rats after Carbon Tetrachloride Poisoning

BY CHISATO HIRAYAMA, KAICHIRO HIROSHIGE AND TOMIICHI MASUYA
*Third Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan*

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1. Collagenolytic activity towards acid-soluble collagen labelled with [$^{14}$C]-proline was assayed in rat liver with and without carbon tetrachloride poisoning. The products of enzymic digestion were found to be free amino acids and peptides. 2. The hepatic collagenolytic activity increased under conditions of single-dose and subacute carbon tetrachloride poisoning, and correlated with hydroxyproline content. The highest activity was found during recovery from subacute poisoning. 3. Under the same experimental conditions, hepatic acid-proteinase activity changed independently of the collagenolytic activity and also of hepatic hydroxyproline content. 4. The increased collagenolytic activity during carbon tetrachloride poisoning was found mainly in the supernatant fraction. 5. The ratio of the collagenolytic activity to hepatic hydroxyproline content increased during recovery from single-dose and subacute poisoning, and decreased during subacute poisoning.

An enzyme system capable of collagen degradation was found in rat liver (Frankland & Wynn, 1962; Wynn, 1967). The enzyme, which originates from lysosomes, has optimum pH 4–5, and a suitable substrate was found to be the acid-soluble collagen, although rat liver lysosomes also solubilize the insoluble collagen at pH 4·0. Unlike cathepsin C activity, the collagenolytic activity is readily lost on moderate heating, and may be chemically separated from cathepsin activity.

A partially purified collagenolytic enzyme, which is not distinguishable from acid proteinase, was reported to be increased in the liver under the conditions of hepatic fibrosis (Bazin & Delaunay, 1964). As in the other mammalian organs, the collagen resorption system in fibrous liver remains to be clarified biochemically. The present investigation attempts to elucidate the significance of the hepatic collagenolytic activity in experimental hepatic injury.

## MATERIALS AND METHODS

*Animals.* Male Wistar strain rats (average body wt. 200 g.), maintained on Clea MF rat diet, were divided into six groups. The group subjected to single-dose poisoning received 0·2 ml. of carbon tetrachloride/100 g. body wt. in mineral oil intraperitoneally. The animals were killed 12 and 48 hr. after the treatment. The group subjected to subacute poisoning received 0·075 ml. of carbon tetrachloride/100 g. body wt. in mineral oil intramuscularly twice weekly. The animals were killed at 1 day after the end of 4, 8 and 12 weeks of the treatment. The group recovering from subacute carbon tetrachloride poisoning was killed at 1 week after the end of 8 weeks of the treatment. The livers were removed and subjected to biochemical analysis.

*Preparation of subcellular fraction.* Rat liver homogenates were fractionated into nuclear, mitochondrial, lysosomal, microsomal and supernatant fractions by the method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). Each fraction was treated with Triton X-100 (final concn. 0·1%) before assay of lysosomal enzymes, including the collagenolytic activity.

*Preparation of $^{14}$C-labelled acid-soluble collagen.* The acid-soluble collagen was prepared by the method of Gallop & Seifter (1963) from rat skin labelled with [$^{14}$C]-proline *in vivo*. Wistar-strain rats (body wt. 100 g.) were injected intraperitoneally with 60 $\mu$c of L-[$^{14}$C]proline (specific radioactivity 8·2 mc/m-mole; the Radiochemical Centre, Amersham, Bucks.) in 0·15 M-NaCl/kg. body wt. and killed after 24 hr. The skin was removed, cleaned from adhering adipose tissue, weighed and homogenized with 0·5 M-sodium acetate. The mixture was stirred mechanically for 18 hr. at 5° and centrifuged. The precipitate was extracted with 75 mM-sodium citrate buffer, pH 3·7, for 18 hr. at 5° and centrifuged at 35000 $g$ for 60 min. The supernatant was filtered through glass wool and dialysed against 20 mM-sodium phosphate and centrifuged. The precipitate was dissolved in and dialysed against 75 mM-sodium citrate buffer, pH 4·0, and centrifuged. The supernatant was dialysed against 20 mM-sodium phosphate and centrifuged. The final precipitate was dissolved in 0·1 M-sodium acetate buffer, pH 4·0, and is referred to as the labelled acid-soluble collagen. The radioactivity was measured with a Beckman DPM 100 liquid-scintillation counter.

*Determination of collagenolytic and glucose 6-phosphatase activities.* The collagenolytic activity was assayed essentially by the method of Frankland & Wynn (1962). Rat livers were homogenized in 0·1M-sodium acetate buffer, pH4·0. A solution (0·5ml.) of the labelled acid-soluble collagen was preincubated at 35°, and mixed with 0·5ml. of 5% liver homogenate. After incubation of the mixture for 18hr. at 35°, 1ml. of ethanol was added. The mixture was kept at room temperature for 30min. and centrifuged at 59000*g* for 20min. at 0°. The radioactivity recovered from the supernatant and the precipitate was almost 100%. The collagenolytic cleavage was determined from the radioactivity of the supernatant expressed as d.p.m./g. of liver. The blank incubation of the substrate resulted in less than 10% of the enzymic cleavage. The collagenolytic activity was assayed in duplicate and expressed as the difference between the enzymic and the non-enzymic cleavage. The liver homogenate, treated with ultrasonics for 2–3min. at 0°, did not show any increased activity, indicating that the particle-bound activity was all released in the present assay. Acid proteinase was determined by the method of Anson (1937), with denatured haemoglobin as substrate. Before enzyme assay, the liver specimen was frozen and thawed. The results are expressed as m-equiv. of tyrosine. Glucose 6-phosphatase activity was determined by the method of Swanson (1955). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with human serum, checked by Kjeldahl nitrogen determination as a standard. Another portion of liver homogenate, hydrolysed in 6M-HCl at 105° for 16hr. was used for hydroxyproline assay.

*Analysis of degradation products of* $^{14}C$*-labelled acid-soluble collagen.* To analyse the enzymic degradation products of the labelled acid-soluble collagen, the substrate, the chemically hydrolysed substrate and the reaction mixture of substrate and liver homogenate were subjected to gel filtration on a column (35cm. × 1·7cm.) of Sephadex G-25. For equilibration and elution 0·1M-sodium acetate buffer, pH4·0, was used.

*Fractionation and determination of proline and hydroxyproline.* The purification and fractionation of proline and hydroxyproline from the collagen hydrolysate were done by the method of Levine (1959). Hydroxyproline and proline were determined by the methods of Troll & Lindsley (1955) and Prockop & Udenfriend (1960) respectively.

## RESULTS

*Properties of* $^{14}C$*-labelled acid-soluble collagen and its degradation products.* The specific radioactivity of the acid-soluble collagen was 2620d.p.m./mg. of protein. The labelled acid-soluble collagen was further hydrolysed in 6M-hydrochloric acid at 105° for 16hr. The hydrolysate was neutralized, purified and fractionated into proline and hydroxyproline, and the content and radioactivity of each amino acid were determined. The specific radioactivity was 6860d.p.m./mg. of proline and 5930d.p.m./mg. of hydroxyproline respectively.

The gel-filtration diagrams of the substrate, the hydrolysed substrate and the reaction mixture of substrate and liver homogenate are illustrated in Fig. 1. The reaction mixture is mainly composed of substrate, degraded substrate and an intermediate component. The intermediate part, eluted in 45–65ml., was characterized as being composed of peptides, which are only enzymically produced.

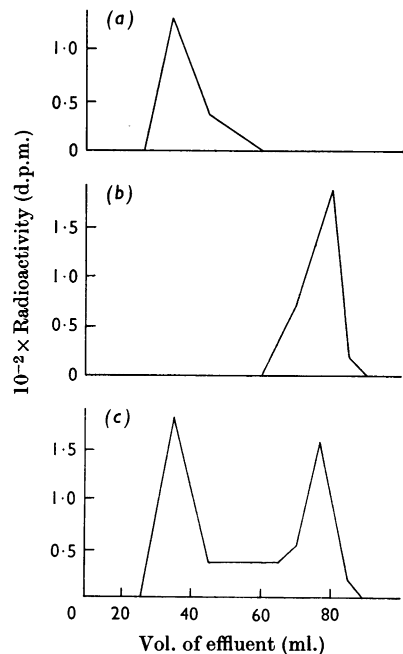


Fig. 1. Elution patterns of $^{14}C$-labelled acid-soluble collagen (*a*), chemically hydrolysed acid-soluble collagen (*b*) and reaction mixture of acid-soluble collagen and rat liver homogenate (*c*) on Sephadex G-25 columns (1·5cm. × 35cm.).

*Changes of glucose 6-phosphatase, acid proteinase and collagenolytic activities in rat liver during carbon tetrachloride poisoning.* The changes of glucose 6-phosphatase, acid-proteinase and collagenolytic activities and hydroxyproline content in the liver as a result of carbon tetrachloride poisoning are shown in Table 1. In hepatic injury glucose 6-phosphatase activity decreased, acid-proteinase activity fluctuated and collagenolytic activity generally increased.

In single-dose carbon tetrachloride poisoning collagenolytic activity increased, whereas acid-proteinase activity decreased. In subacute carbon tetrachloride poisoning collagenolytic and acid-proteinase activities generally increased. The former seemed to change in parallel with hydroxyproline content. The highest collagenolytic activity was found in the animals recovering from subacute poisoning, in which fibre resorption occurred most actively. In this condition, acid-proteinase activity did not change significantly.

Table. 1. *Changes in glucose 6-phosphatase, acid-proteinase and collagenolytic activities and hydroxyproline content in rats as a result of carbon tetrachloride poisoning*

The treatment of animals and the assay methods are described in the text. Results are given as means ± S.D. Significant differences from control group values are marked: * ($P<0.05$); ** ($P<0.01$). Significant differences between the subacute poisoning (8 weeks) group and recovery groups are marked: † ($P<0.01$).

| Group | No. of rats | Glucose 6-phosphatase activity (mg. of $P_i$/g. of liver | $10^{-5}\times$ Acid proteinase activity (m-equiv. of tyrosine/g. of liver) | $10^{-3}\times$ Collagenolytic activity (d.p.m./g. of liver) | Hydroxyproline content (µg./g. of liver) | Collagenolytic activity/hydroxyproline content |
|---|---|---|---|---|---|---|
| Control | 8 | 4·21±0·19 | 33·6± 8·5 | 3·86±0·61 | 139± 24 | 28·4± 4·5 |
| Single-dose poisoning | | | | | | |
| 12hr. | 5 | 1·04±0·20** | 21·4± 2·6** | 6·65±2·98** | 184± 14** | 35·5±13·7 |
| 48hr. | 6 | 1·54±0·38** | 18·5± 2·2** | 6·20±1·05** | 174± 19* | 38·0± 5·5* |
| Subacute poisoning | | | | | | |
| 4 weeks | 5 | 2·34±0·20** | 50·7± 9·5** | 8·14±2·62** | 418±102** | 21·9±13·0 |
| 8 weeks | 4 | 2·04±0·66** | 136·1±15·0** | 9·96±0·87** | 448± 53** | 22·3± 5·8 |
| Recovery (1 week) | 4 | 2·60±0·56** | 26·8± 5·6† | 13·02±1·24**† | 393± 80** | 36·8±10·6 |
| 12 weeks | 5 | 1·49±0·26** | 45·1± 9·9** | 11·24±2·22** | 636±252** | 19·1± 5·7* |

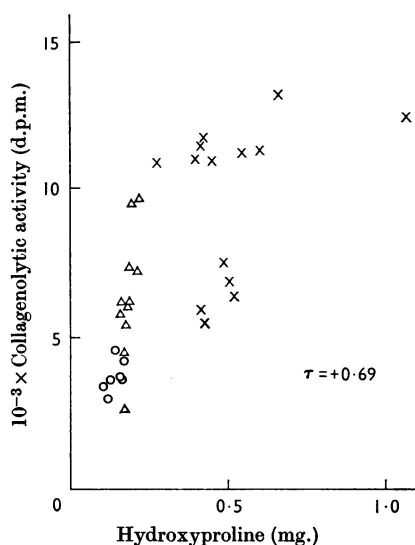


Fig. 2. Relationship between collagenolytic activity and hydroxyproline content in rat liver during carbon tetrachloride poisoning. ○, Control; △, single-dose poisoning; X, subacute poisoning.

To clarify the role of the collagenolytic activity in hepatic collagen metabolism, the relationship between collagenolytic activity and hydroxyproline content was studied under the conditions of single-dose and subacute carbon tetrachloride poisoning. As shown in Fig. 2, the activity correlated with hydroxyproline content significantly (correlation coefficient + 0·69), although acid proteinase activity did not so do. To assess the relative capacity for removing collagen in the liver, the ratio of collagenolytic activity to hydroxyproline content was calculated (Table 1). The ratio increased in the livers of animals recovering from single-dose and subacute carbon tetrachloride poisoning, but decreased in the late period of subacute poisoning.

*Intracellular distribution of collagenolytic activity.* Table 2 shows the intracellular distribution of collagenolytic activity in rat liver with and without carbon tetrachloride poisoning. In normal rat liver the highest specific activity was found in the lysosomal fraction. With subacute carbon tetrachloride poisoning the activity increased in both the lysosomal and supernatant fractions, and the specific activity in the supernatant fraction was nine times the control value. These results indicate that the increased activity was mainly in the supernatant fraction rather than the lysosomes.

## DISCUSSION

The exact mechanism of collagen degradation in the liver remains obscure. As suggested from studies on the degradation of $^{131}$I-labelled albumin (Mego & McQueen, 1965; Mego, Bertini & McQueen, 1967), the collagen such as the soluble collagen, which can be detected in blood plasma (LeRoy, Kaplan, Udenfriend & Sjoerdsma, 1964), may first be taken up within small pinocytic vesicles and then transported to lysosomes. Because of the low pH believed to exist within lysosomes, the intralysosomal collagen enters an acid medium and suffers denaturation before digestion occurs. In addition, 35° is close to the critical temperature for collagen (Evanson, Seffrey & Krane, 1967), so that the acid-soluble collagen used as the substrate could

Table 2. *Intracellular distribution of hepatic collagenolytic activity in rats with and without subacute carbon tetrachloride poisoning*

Each group consists of three animals. The subacute-poisoning group was treated with carbon tetrachloride for 8 weeks. Methods for the fractionation and the assay are described in the text.

| | Nuclei | Mitochondria | Lysosomes | Microsomes | Supernatant |
|---|---|---|---|---|---|
| Control | | | | | |
| Protein (mg./g. of liver) | 31 | 26 | 22 | 37 | 63 |
| Activity (d.p.m./g. of liver) | 117 | 389 | 1383 | 300 | 1117 |
| Specific activity (d.p.m./mg. of protein) | 4 | 15 | 63 | 8 | 18 |
| Subacute poisoning | | | | | |
| Protein (mg./g. of liver) | 28 | 17 | 16 | 26 | 39 |
| Activity (d.p.m./g. of liver) | 289 | 181 | 2764 | 343 | 6350 |
| Specific activity (d.p.m./mg. of protein) | 10 | 11 | 173 | 13 | 163 |

be easily denatured by lysosomes. Because the hepatic collagenolytic acitivity is very weak at neutral pH (Woods & Nichols, 1965), it seems likely that hepatic collagenolysis would proceed *in vivo* under conditions similar to those of the present assay. As with other protein substrates (Coffey & de Duve, 1968), the main enzymic products of the degradation collagens by lysosomes were free amino acids and peptides, which may be further degraded to amino acids by peptidase (Woessner, 1962).

Several enzymes of liver have been suggested as collagen-degrading systems. A lysosomal collagenolytic activity, which differs from true collagenase (EC 3.4.4.19), is believed to be an acid proteinase (Bazin & Delaunay, 1964). According to Frankland & Wynn (1962) and Wynn & Wahid (1966), however, the activity could be distinguished from acid proteinase by acetone fractionation or by moderate heating. The activity may act on ester linkages or on the telopeptide region. In the present work also the hepatic collagenolytic activity was distinct from acid-proteinase activity because it increased markedly in the recovery from hepatic fibrosis, whereas acid-proteinase activity decreased or did not change significantly. The results provide further evidence that the collagenolytic activity is at least not identical with acid proteinase, but seems to be related to collagen-resorption phenomena in the liver.

In hepatic injury hepatic collagen synthesis is generally accelerated. Labelling studies *in vitro* with human liver (Hirayama, Kimura, Irisa & Masuya, 1968) proved that the specific radioactivity of the hot-trichloroacetic acid-soluble fraction of liver increased in patients with chronic liver disease, especially with chronic active hepatitis. Protocollagen hydroxylase activity also increased in the rat liver with subacute carbon tetrachloride poisoning (Takeuchi, Kivirikko & Prockop, 1967). The present investigation shows that hepatic collagenolytic activity generally increased in liver injury, suggesting that the hepatic collagenolytic enzyme seems to participate in collagen metabolism as a homoeostatic mechanism.

The present investigation showed that the increased collagenolytic activity was found mainly in the hepatic supernatant fraction. This could either result from the activity being released from lysosomes or from the activity being induced under this experimental condition. It is generally accepted that hepatotoxins cause a release of lysosomal enzymes into the cell sap or the surrounding space (Weissmann, 1965). In fact, *N*-acetyl-$\beta$-glucosaminidase activity increased in the serum and liver supernatant fraction during subacute carbon tetrachloride poisoning (Koizumi, Suematsu, Iwabori & Abe, 1968). It seems, however, more likely that the activity was induced under the present conditions, because the activity increased in both the lysosomes and the supernatant fraction. We cannot exclude the possibility that the cell-fractionation procedure caused a release of some particle-bound enzymes into the supernatant fraction, especially with liver subjected to carbon tetrachloride poisoning.

From morphological studies (Barka, Schaffner & Popper, 1961; Rubin, Hutterer & Popper, 1963), fibre absorption may arise also from mesenchymal cells, which appear mainly in the period of recovery from hepatic fibrosis and also from non-septal hepatic fibrosis. Houck & Sharma (1968) have reported that fibroblasts excrete collagenolytic activity extracellularly under some conditions. If some mesenchymal cells possess such collagenolytic activity, the excreted activity should be recovered

in the supernatant fraction, as shown by the present study.

Irrespective of the origin, at the recovery stage from single-dose and subacute carbon tetrachloride poisoning the ratio of hepatic collagenolytic activity to hydroxyproline content increased, suggesting that the fibre suffers a rapid degradation and disappears. In subacute carbon tetrachloride poisoning, morphologically regarded as the non-septal hepatic fibrosis, the synthesis and degradation of collagens take place actively. During 12 weeks of treatment with carbon tetrachloride, however, hepatic collagen content increased rapidly, so that septal fibrosis was developing. At this time the hepatic collagenolytic activity seems not to be sufficient to remove the synthesized collagen, as seen in the decreased ratio of the collagenolytic activity to the hydroxyproline content. The results provide evidence that the relative lack of fibre degradation is also responsible for septum formation at the precirrhotic and cirrhotic stages.

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