Quantitative and Metabolic Changes of Hepatic Collagens in Rats after Carbon Tetrachloride Poisoning

BY CHISATO HIRAYAMA, IKUO MOROTOMI AND KAICHIRO HIROSHIGE
Third Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka, Japan

(Received 17 November 1969)

1. The collagen hydroxyproline in rat liver was composed of 3.5% neutral-soluble collagen, 4.9% acid-soluble collagen and 91.6% insoluble collagen. In labelling studies with [^{14}C]proline *in vitro*, the specific radioactivities of neutral-soluble, acid-soluble and insoluble collagens in rat liver were found to be 233 000, 69 000 and 830 d.p.m./ μmol of hydroxyproline respectively after 1 h. 2. During subacute carbon tetrachloride poisoning the hepatic content of insoluble collagen markedly increased, whereas those of soluble collagens did not change. During recovery from subacute poisoning hepatic contents of soluble collagens were markedly decreased. 3. After 8 weeks of carbon tetrachloride poisoning the specific radioactivities of hepatic soluble collagens increased, while that of insoluble collagen decreased. During recovery from subacute poisoning, the specific radioactivities of soluble collagens decreased to the normal range and that of insoluble collagen further decreased. 4. Hepatic collagenolytic activity solubilizing insoluble collagen, which differs from mammalian collagenase, decreased under the conditions of the subacute poisoning and also during recovery from subacute poisoning.

Initial studies on hepatic collagen revealed that hepatic insoluble collagen turned over slowly compared with hepatic non-collagen proteins (Neuberger, Perrone & Slack, 1951; Neuberger & Slack, 1953; Robertson, 1952). Later investigation, however, indicated that hepatic collagens including soluble collagens turned over rapidly (Gerber, Gerber & Altman, 1960). In experimental hepatic injuries or in human chronic liver diseases the specific radioactivity of hepatic collagen generally increased, though that of the insoluble collagen occasionally decreased (Hirayama, Fukuda, Tominaga & Masuya, 1963; Hirayama, Kimura, Irisa & Masuya, 1968). Although hepatic fibrosis appears as a repair process after injury, no exact information on hepatic collagen metabolism under the conditions of hepatic injury has been obtained so far. The present investigation attempts to elucidate quantitative as well as metabolic changes of hepatic collagens in experimental hepatic injury.

MATERIALS AND METHODS

Animals. Male Wistar rats (average body wt. 200 g), maintained on Clea MF rat diet, were divided into four groups. 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 1 day after the end of 4, 8 and 12 weeks of the treatment. The group recovering from subacute carbon

tetrachloride poisoning was killed 1 week after the end of 8 weeks of the treatment. The livers were removed and subjected to biochemical analysis.

Preparation and determination of hepatic collagens. The fractionation of hepatic collagens was done essentially by the method of Jackson (1957). Rat livers were homogenized in 9 vol. of 0.2 M-NaCl, pH 7.4. The mixture was stirred mechanically for 24 h at 4°C and centrifuged. The supernatant is referred to as the neutral-soluble collagen. The precipitate was extracted with 0.2 M-sodium citrate buffer, pH 3.5, for 24 h at 4°C and centrifuged. The supernatant and the precipitate are referred to as the acid-soluble collagen and the insoluble collagen respectively. The liver homogenate and each collagen fraction, precipitated and washed with 10% (w/v) trichloroacetic acid, were further extracted with 5% (w/v) trichloroacetic acid at 90°C for 30 min (Fitch, Harkness & Harkness, 1955), followed by centrifugation. The supernatant fraction was hydrolysed in 6 M-HCl at 105°C for 16 h in a sealed tube. Hydroxyproline content was determined by the method of Stegemann & Stalder (1967).

Determination of specific radioactivities of hepatic collagens. A 1 g portion of liver slices was incubated in 10 ml of the medium (Marsh & Drabkin, 1958) containing 10 μCi of L-[^{14}C]proline (The Radiochemical Centre, Amersham, Bucks., U.K.) under an atmosphere of $\text{O}_2 + \text{CO}_2$ (95:5) at 37°C for 0, 30, 60 and 120 min; usually the incubation was for 60 min. The labelled liver, after the incubation medium had been removed on a filter paper, was homogenized and fractionated into three collagen fractions as described by Jackson (1957). Hydroxyproline was isolated from the hydrolysed collagen fractions by the method of Peterkofsky & Prockop (1962), and

Table 1. *Changes in hepatic contents of collagens in rats with subacute carbon tetrachloride poisoning*

The treatment of animals and the assay methods are described in the text. Results are given as means $\pm$ S.D. in units of μg of hydroxyproline/g of liver. Significant differences from control-group values are marked: * $P < 0.05$; ** $P < 0.01$. Significant differences between the 8-weeks group and the recovery group are marked: † $P < 0.05$; †† $P < 0.01$.

Group	No. of rats	Neutral-soluble collagen	Acid-soluble collagen	Insoluble collagen	Recovery (%)
Control	5	5.0 $\pm$ 0.9	7.1 $\pm$ 3.7	132 $\pm$ 22	105 $\pm$ 10
CCl ₄ poisoned					
4 weeks	5	8.1 $\pm$ 3.0	11.3 $\pm$ 1.3*	458 $\pm$ 53**	105 $\pm$ 9
8 weeks	5	6.0 $\pm$ 2.2	5.1 $\pm$ 1.0	550 $\pm$ 123**	124 $\pm$ 32
Recovery					
1 week	5	3.1 $\pm$ 1.0*†	0.8 $\pm$ 0.7**††	422 $\pm$ 79**	95 $\pm$ 14
12 weeks	5	8.1 $\pm$ 4.0	9.0 $\pm$ 2.0	754 $\pm$ 345**	119 $\pm$ 14

Fig. 1. Time curves of specific radioactivities of collagen fractions in rat liver *in vitro*. ○, Neutral-soluble collagen; △, acid-soluble collagen; ●, insoluble collagen.

hydroxyproline content was determined as described by Stegemann & Stalber (1967). The radioactivity was measured with a Beckman DPM100 liquid-scintillation counter. The specific radioactivity of each collagen fraction was determined, and then that of total collagen was calculated.

Determination of collagenolytic activity towards insoluble collagen. The collagenolytic activity towards insoluble collagen was assayed essentially by the same method as that using the labelled acid-soluble collagen as a substrate (Frankland & Wynn, 1962; Hirayama, Hiroshige & Masuya, 1969). The activity, which is distinguishable from a specific mammalian collagenase (Gross, 1964),

has been reported as a non-specific enzyme system in lysosomes. The insoluble collagen was obtained from rat skin labelled with [¹⁴C]proline *in vivo*. The insoluble collagen fraction, washed several times with water, was homogenized, kept at -20°C overnight and freeze-dried. The freeze-dried substrate was stored at -20°C until required. The specific radioactivity was 1993 d.p.m./mg of protein, compared with 2620 d.p.m./mg of protein for acid soluble collagen. Rat livers were homogenized in 0.1 M-sodium acetate buffer, pH 4.0. The liver homogenate, treated with ultrasonics for 2–3 min at 0°C , did not show any increased activity, indicating that the particle-bound activity was all released in the present assay. A 10 mg portion of the substrate was mixed with 1 ml of 10% liver homogenate. After incubation for 18 h at 35°C the mixture was centrifuged at 120000g for 30 min at 0°C . The collagenolytic activity towards insoluble collagen was determined from the radioactivity of the supernatant, expressed as d.p.m./g of liver.

RESULTS

Changes of hepatic collagens in rats during carbon tetrachloride poisoning. As shown in Table 1, the hepatic collagen in rats, expressed as hydroxyproline, was composed of 3.5% neutral-soluble collagen, 4.9% acid-soluble collagen and 91.6% insoluble collagen. The recovery of collagen fractions was found to be 105 $\pm$ 10% of the total hepatic collagen. During the subacute carbon tetrachloride poisoning the hepatic content of insoluble collagen markedly increased, whereas those of soluble collagens did not change. Thus an increased content of the insoluble collagen is responsible for the increase in hepatic collagen content as a result of liver injury. In rats recovering from subacute poisoning decreased contents of soluble collagens were noticed, suggesting that soluble collagens could be absorbed more easily than insoluble collagen. These findings indicate also that hepatic collagen under the conditions of carbon tetrachloride poisoning is characterized by having less of the soluble collagen than normal collagen.

Table 2. *Changes in specific radioactivities of hepatic collagens and collagenolytic activity towards insoluble collagen in rats with carbon tetrachloride poisoning*

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

Group	No. of rats	$10^{-3} \times \text{Sp. radioactivity (d.p.m./}\mu\text{mol of hydroxyproline)}$				No. of rats	$10^{-3} \times \text{Collagenolytic activity (d.p.m./g of liver)}$
		Collagen	Neutral-soluble collagen	Acid-soluble collagen	Insoluble collagen		
Control	5	8.8 ± 1.5	233 ± 96	69 ± 38	0.83 ± 0.16	5	27.6 ± 3.3
CCl_4 poisoned 8 weeks	5	8.6 ± 0.4	$772 \pm 109^{**}$	$461 \pm 31^{**}$	$0.50 \pm 0.04^*$	5	$20.7 \pm 2.7^{**}$
Recovery 1 week	5	$3.6 \pm 1.0^{**\dagger\dagger}$	$421 \pm 206^{\dagger\dagger}$	$61 \pm 57^{\dagger\dagger}$	$0.21 \pm 0.07^{**\dagger\dagger}$	4	$23.2 \pm 2.6^*$

Changes of specific radioactivities of hepatic collagens in carbon tetrachloride-poisoned rats. Time curves of the specific radioactivities of hepatic collagens of rats *in vitro* are shown in Fig. 1. There was no significant labelling at zero time and an approximately linear increase of the labelling was noticed up to 120 min. Although the short incubation period employed in the present investigation might label only soluble collagens (Jackson, 1957), the present experiment showed that the insoluble collagen could also be labelled *in vitro*. As shown in Table 2, the specific radioactivities (d.p.m./ μmol of hydroxyproline) of hepatic collagens in control rats were found to be 233 000 for neutral-soluble collagen, 69 000 for acid-soluble collagen and 830 for insoluble collagen after 1 h. After 8 weeks of carbon tetrachloride poisoning the specific radioactivities of soluble collagens markedly increased, while that of insoluble collagen significantly decreased. During recovery from subacute poisoning the increased specific radioactivities of soluble collagens decreased towards the normal range, and that of insoluble collagen further decreased. The results indicate that carbon tetrachloride poisoning causes a rapid response in the synthesis of hepatic soluble collagens, but not of hepatic insoluble collagen, which seems to be metabolically inert even in this condition.

Changes of hepatic collagenolytic activity towards insoluble collagen in carbon tetrachloride poisoning. As shown in Table 2, 1 g of rat liver can solubilize insoluble collagen *in vitro* at a rate of 27 600 d.p.m./18 h, or 0.77 mg/h. The collagenolytic activity decreased after 8 weeks of carbon tetrachloride poisoning and also in its resorption period. Therefore the collagenolytic activity towards insoluble collagen decreases as a result of hepatic injury, so that the enzyme activity seems not to be identical with the collagenolytic activity towards the acid-

soluble collagen, which increases under the same experimental conditions (Hirayama *et al.* 1969).

DISCUSSION

There is little information about biochemical parameters concerning hepatic collagens. The present investigation proved that 92% of the collagen in rat liver was insoluble collagen. The result is similar to the composition of rat skin collagen (Houck & Patel, 1965). The ratios of specific radioactivities of hepatic collagens were also almost identical with those of rat skin collagens labelled *in vivo* for 2 h. Thus the nature of hepatic collagen is also characterized as one of the common body collagens. From morphological studies the cell lines responsible for the collagen synthesis in liver during carbon tetrachloride poisoning are fibroblasts associated with ductular cells (Rubin, Hutterer & Popper, 1963; Stenger, 1965).

The present investigation showed that the specific radioactivities of hepatic soluble collagens were raised in this condition. Because hepatic collagenolytic activity towards acid-soluble collagen also increased, hepatic soluble collagens should turn over rapidly under the conditions of hepatic injury, so that a significant amount of soluble collagens are rapidly lost before undergoing cross-linking. In this condition, however, the specific radioactivities of soluble collagens increased more markedly than does the collagenolytic activity towards acid-soluble collagen, and therefore excess of soluble collagens seems to enter the insoluble collagen pool, where the insoluble collagen turns over more slowly, and causes the fibrous liver. In fact, the collagenolytic activity towards insoluble collagen was decreased. These results are similar to previous findings (Hirayama *et al.* 1963, 1968).

During hepatic fibre resorption the specific radioactivities of hepatic collagens generally decreased compared with those during subacute carbon tetrachloride poisoning (Table 2). On the other hand, the collagenolytic activity towards acid-soluble collagen was markedly raised (Hirayama *et al.* 1969), though the activity towards insoluble collagen was less than in the poisoned condition. These findings suggest that the turnover of the soluble collagens was accelerated, but not that of the insoluble collagen. Hepatic contents of soluble collagens were found to be markedly decreased during recovery (Table 1). The metabolically inert insoluble collagen is deposited in the damaged liver and continues to be so for 1 week of the resorption period.

In contrast with the catabolism of soluble collagens, the breakdown mechanism of insoluble collagen *in vivo* remains to be clarified. Turnover studies on catabolized collagen products in urine, however, point to a hydrolysis of the peptide chains of body collagens, including insoluble collagen (Prockop, 1964). Some collagenase activity at neutral pH was found to be present extracellularly in bone and skin tissue (Woods & Nichols, 1965; Eisen, Jeffrey & Gross, 1968). Therefore it is reasonable to assume that hepatic insoluble collagen is at first modified by the extracellular collagenase and then taken up within pinocytic vesicles and transported to lysosomes. Thus an enzyme system that solubilizes insoluble collagen at acid pH values was found in rat liver lysosomes (Wynn, 1967; Anderson, 1969), where the solubilized collagen was further degraded by the collagenolytic activity towards acid-soluble collagen. It seems likely that three enzyme systems take part in degrading hepatic insoluble collagen *in vivo*. In addition, some proteolytic enzymes should participate also in the catabolic system for collagens.

As with the soluble collagens, the decreased capacity to degrade insoluble collagen should be the key step for septum formation. At present, however, it remains obscure which lysosomal collagenolytic enzyme is really responsible for the fibre resorption in damaged liver. For example, from previous and present results, an assumption was made that 1g of rat liver was capable of solubilizing 0.77 mg of

insoluble collagen/h and of degrading 0.12 mg of acid-soluble collagen/h. As a result of carbon tetrachloride poisoning, 1g of rat liver was capable of solubilizing 0.58 mg of insoluble collagen/h and of degrading 0.31 mg of acid-soluble collagen/h. Therefore, if the collagenolytic activity towards acid-soluble collagen is the only intracellular degradation system for soluble collagens, the activity may be rate-limiting for the fibre resorption process in liver cells.

REFERENCES

- Anderson, A. J. (1969). *Biochem. J.* **113**, 457.
 Eisen, A. Z., Jeffrey, J. J. & Gross, J. (1968). *Biochim. biophys. Acta*, **151**, 637.
 Fitch, S. M., Harkness, M. L. R. & Harkness, R. D. (1955). *Nature, Lond.*, **177**, 163.
 Frankland, D. M. & Wynn, C. H. (1962). *Biochem. J.* **85**, 276.
 Gerber, G., Gerber, G. & Altman, K. (1960). *J. biol. Chem.* **235**, 2653.
 Gross, J. (1964). *Biophys. J.* **4** (Suppl.), 63.
 Hirayama, C., Fukuda, T., Tominaga, K. & Masuya, T. (1963). *Acta hepato-splenol.* **10**, 154.
 Hirayama, C., Hiroshige, K. & Masuya, T. (1969). *Biochem. J.* **115**, 843.
 Hirayama, C., Kimura, N., Irisa, T. & Masuya, T. (1968). *Clinica chim. Acta*, **21**, 49.
 Houck, J. C. & Patel, Y. M. (1965). *Nature, Lond.*, **206**, 158.
 Jackson, D. S. (1957). *Biochem. J.* **65**, 227.
 Marsh, J. B. & Drabkin, D. L. (1958). *J. biol. Chem.* **230**, 1073.
 Neuberger, A., Perrone, J. C. & Slack, G. B. (1951). *Biochem. J.* **49**, 199.
 Neuberger, A. & Slack, H. G. B. (1953). *Biochem. J.* **53**, 47.
 Peterkofsky, B. & Prockop, D. J. (1962). *Analyt. Biochem.* **4**, 400.
 Prockop, D. J. (1964). *J. clin. Invest.* **43**, 453.
 Robertson, W. B. (1952). *J. biol. Chem.* **197**, 495.
 Rubin, E., Hutterer, F. & Popper, H. (1963). *Am. J. Path.* **42**, 715.
 Stegemann, H. & Stalber, K. (1967). *Clinica chim. Acta*, **18**, 267.
 Stenger, R. D. (1965). *Expl molec. Path.* **4**, 357.
 Woods, J. F. & Nichols, G., jun. (1965). *J. Cell Biol.* **26**, 747.
 Wynn, C. H. (1967). *Nature, Lond.*, **215**, 1191.