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Changes in prolinase and prolidase activity during CCl_4 administration inducing liver cytolysis and fibrosis in rat

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Summary. In earlier papers, we reported that the activity of prolidase (EC 3.4.13.9) increased in the plasma of patients with cirrhosis, while that of serum prolinase (EC 3.4.13.8) was normal and was affected only by necrosis. In this work, we investigated prolinase and prolidase activity during short and long-term CCL_4 administration in the rat. After a single dose, prolinase activity increased in serum faster than did prolidase activity and it also decreased more slowly. Within the liver, no significant change in these two enzyme activities was observed during the acute phase of necrosis. During chronic CCl_4 intoxication, the rises in prolidase and prolinase activity in rat serum were difficult to interpret, because of the liver necrosis present throughout the experiment. However, within the liver, prolinase activity was not affected, unlike that of prolidase which rose at week 3, reached a maximum value at week 6 (reversible fibrosis) and remained elevated at weeks 10 and 12 (irreversible fibrosis). The increase in prolidase activity was specific for liver and was not observed in other tissues. These results are in agreement with those obtained in humans; they highlight the possible physiological significance of enhanced liver prolidase activity during the fibrotic process.

Keywords: prolinase, prolidase, carbon tetrachloride, cytolysis, fibrosis, liver

CCl_4-treated rat is the experimental model most often used to study liver fibrosis and is the best documented with regard to collagen synthesis and degradation (for recent reviews, Rojkind & Perez Tamayo 1983; Perez-Tamayo 1983; Last 1985). The activity of enzymes involved in intracellular post-translational modifications of collagen increases (Risteli & Kivirikko 1974) and so does lysyloxidase activity (Siegel *et al.* 1978) which permits aldehyde formation. Extracellular collagen degradation is well documented in the CCl_4 model (Hirayama *et al.* 1969; Okazaki & Maruyama 1974; Montfort & Perez-Tamayo 1978; Carter *et al.* 1982; Lindblad & Fuller 1983) but, to our knowledge, few experiments have been published concerning intracellular procollagen catabolism.

Prolinase (EC 3.4.13.8) and prolidase (EC 3.4.13.9) are two enzymes which split dipeptides containing proline or hydroxyproline at the C- and N-termini respectively. The physiological importance of these two dipeptidases is unknown. They could be involved in the inactivation of a large number of neurological peptide hormones (Myara *et al.* 1984*a*) and also probably in the last step of intracellular procollagen degradation since it contains a large amount of proline and

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hydroxy-4 proline. Our initial studies centered on prolidase deficiency (for review see Myara *et al.* 1984*a*), a genetic disease inherited as an autosomal recessive trait characterized by chronic recurrent ulcers with massive iminodipeptiduria. In prolidase-deficient cultured skin fibroblasts, collagen hydroxylation was normal (Myara *et al.* 1983; Royce & Danks 1982), the proline pool diminished markedly and intracellular collagent degradation was increased (Myara *et al.* 1983). Since changes in collagen metabolism are induced by prolidase deficiency, this enzyme's activity in syndromes involving a collagen disorder such as chronic liver disease was explored in an earlier study; prolidase activity increased in the plasma of patients with cirrhosis (Myara *et al.* 1984*b*). It also increased in the acute phase of liver necrosis (Myara *et al.* 1985) but fell rapidly to normal before aminotransferase activity. Using the CCL_4 rat model, Zuyderhoudt *et al.* (1985) observed no increase in plasma prolidase activity after 3 or 5 weeks of treatment but they did not investigate the enzyme changes associated with irreversible cirrhosis, nor did they determine liver prolidase activity. Before investigating further the prolidase activity in CCl_4-treated rats, it seemed to be of more interest to study the other dipeptidase; i.e. prolinase for which no deficiency has been described. Recent studies in our laboratory showed that this enzyme's activity is strongly linked with liver necrosis and remains virtually normal in cirrhotic patients with enhanced prolidase activity (Myara *et al.* 1985).

In the present study, the activities of prolidase and prolinase during experimental necrosis, and reversible and irreversible cirrhosis of the rat liver are investigated.

Materials and methods

Animals. Male Wistar rats were obtained from Iffa Credo (France). The animals were housed under a 12 h light/12 h dark cycle and fed on a commercial standard diet (AO_4-UAR). They were allowed food and water *ad libitum.*

Acute CCL_4 intoxication. Three experiments were carried out.

Experiment 1. 12 rats weighing 200–250 g were given a single dose of CCl_4 i.p. (0.30 ml/100 g 50% vol/vol in olive oil) and three rats were killed at 1, 3, 6 and 16 h after injection. Blood was taken by cardiac exsanguination under ether anaesthesia, and the activity of serum prolidase, prolinase, alanine and aspartate aminotransferase was determined.

Experiment 2. A single dose of CCl_4 was administered i.p. to nine rats weighing 200–250 g and blood was taken from the tail of each rat on days 1, 2, 3, 4, and 7 thereafter.

Experiment 3. A single dose of CCl_4 was injected in six rats weighing 100–110 g and liver was removed two days after the injection.

Chronic CCl_4 intoxication. Seventy rats weighing 100–110 g received 2 weekly i.p. injections of CCl_4 (0.30 ml/100 g, 50% vol/vol in olive oil) for 3, 6, 10 or 12 weeks.

Animals were killed 72 h after the last injection. The liver was removed and immediately placed on ice and a portion was then immersed in Bouin's fixative for histological examination. At week 12, other tissues (kidney, ileum, jejunum duodenum, colon, pancreas, stomach, lung, spleen, adrenal gland, brain, cerebellum, heart, testis, skeletal muscle, skin) were also removed and treated in the same way as the liver.

Two control groups were also studied. The rats in the first group ($n=18$) were kept without any treatment, and those in the second group ($n=11$) received 2 weekly i.p. injections of olive oil. Six control rats were killed at weeks 0, 3, 6 and 10, and five others, at week 12.

Histological examination. The fixed samples were embedded in paraffin with 10% pycolite by routine procedures. Each specimen was step-sectioned and stained with haemalum-eosin-sufran, trichrome, and also examined by the Gordon-Sweet and Perls techniques.

The slides were studied by a pathologist

with no previous knowledge of the activities of the enzymes explored.

Activity of serum prolinase, prolidase, aspartate and alanine aminotransferase. Serum was separated by centrifugation at 2300 *g* at 4°C for 15 min and stored at 4°C. Enzyme activities were tested within 24 h of serum collection.

To test prolinase activity (Myara *et al.* 1985), 0.1 ml of serum was incubated at 37°C for 90 min with 0.1 ml of 0.3 mol/l tris/HCl buffer pH 9.0 containing 0.06 mmol/l $MnCl_2$ (final concentration: 0.02 mmol/l) and 0.1 ml of 100 mmol/l prolyl-L-valine (Sigma) prepared in water. The reaction was stopped with 1 ml of 0.45 mol/l trichloroacetic acid, and the supernatant was used for proline estimation as previously described (Myara *et al.* 1982).

To test prolidase activity (Myara *et al.* 1984b), serum was diluted six-fold with 0.050 mol/l tris/HCl buffer pH 7.8 containing 1 mmol/l $MnCl_2$, and preincubated for 24 h at 37°C. 0.1 ml of 94 mmol/l glycyl-L-proline (Sigma) prepared in tris/$MnCl_2$ buffer was then added to an 0.1 ml aliquot of the diluted and preincubated serum. After incubation for 30 min at 37°C, the reaction was stopped by adding 1 ml of 0.45 mol/l trichloroacetic acid, and the supernatant was treated as above.

Aspartate aminotransferase (Mathieu *et al.* 1976) and alanine aminotransferase (Mathieu *et al.* 1978) were routinely determined at 30°C according to the recommendations of the Société Française de Biologie Clinique.

Prolinase and prolidase activity in rat liver. Liver samples were taken from the chilled livers and rinsed with 0.15 mol/l NaCl and homogenized in an ice-bath for 1 min with 0.05 mol/l tris/HCl buffer pH 7.8 (5 ml/0.1 g liver) using an Ultra Turax homogenizer. The homogenate was centrifuged at 200 *g* for 15 min at 4°C and the supernatant was used for the enzyme assays.

Prolidase assay. An aliquot was diluted twice with 0.05 mol/l tris/HCl buffer pH 7.8 containing 2 mmol/l $MnCl_2$ and preincubated for 24 h at 37°C. Liver prolidase activity was then determined in the same way as the serum activity.

Prolinase assay. An aliquot was diluted four-fold with 0.15 mol/l NaCl and the prolinase activity determined as the serum.

Proteins were measured with coomassie brilliant blue (Bio-Rad 1977) using bovine serum albumin as standard.

Results

Enzyme activity during acute CCl_4 intoxication

Experiment 1. After a single i.p. dose of CCl_4 serum prolinase activity was increased within the first hour; prolidase activity, which was still normal at hour 1, had risen by hour 3 (Table 1).

Experiment 2. To simplify data presentation, we pooled the values for the nine rats studied (Table 1). The maximum prolidase and prolinase activities were obtained between days 1 and 2. Prolidase activity decreased and fell to below the normal value on day 3, whereas prolinase activity was always enhanced until after day 7. The modifications observed for prolinase were parallel to those of asparate and alanine aminotransferase.

Experiment 3. Two days after a single injection of CCl_4, no significant modification of prolidase or prolinase activity was observed in liver (Table 2).

Enzyme activities during chronic CCl_4 intoxication

In serum, prolidase, prolinase and amino transferase activities increased throughout the experiment (Table 1).

In liver, prolinase activity fluctuated around the normal value, while prolidase activity rose at week 3, reached its maximum at weeks 6 and 10, and then slowly declined at week 12 (Table 2).

Prolidase activity in other tissues was normal in rats treated with CCl_4 for 12

Table 1. ASAT, ALAT, prolidase and prolinase activity in rat serum during CCl_4 intoxication

	ASAT U/l mean ± s.e.m	ALAT U/l mean ± s.e.m.	Prolidase U/l mean ± s.e.m.	Prolinase U/l mean ± s.e.m.
Control ($n=32$)	71 ± 2	35 ± 1	2450 ± 40	90 ± 3
Acute intoxication				
Experiment 1				
hour 1 ($n=3$)	153 ± 25	95 ± 13	2310 ± 130	220 ± 6
hour 3 ($n=3$)	227 ± 78	91 ± 21	3000 ± 190	345 ± 18
hour 6 ($n=3$)	237 ± 72	120 ± 40	2870 ± 30	379 ± 35
hour 16 ($n=3$)	372 ± 35	252 ± 40	3730 ± 120	643 ± 51
Experiment 2				
day 1 ($n=9$)	1300 ± 140	1220 ± 200	3780 ± 230	916 ± 79
day 2 ($n=9$)	1830 ± 380	1570 ± 390	3360 ± 180	1100 ± 140
day 3 ($n=9$)	170 ± 14	150 ± 23	2270 ± 80	540 ± 58
day 4 ($n=9$)	68 ± 6	47 ± 5	2050 ± 30	320 ± 21
day 7 ($n=8$)	82 ± 4	40 ± 3	2080 ± 70	112 ± 7
Chronic intoxication				
week 3 ($n=8$)	400 ± 50	270 ± 35	2855 ± 135	725 ± 70
week 6 ($n=7$)	1550 ± 330	1485 ± 410	3990 ± 210	1455 ± 185
week 10 ($n=5$)	860 ± 210	620 ± 200	3110 ± 275	1190 ± 235
week 12 ($n=5$)	605 ± 185	240 ± 90	2890 ± 185	840 ± 90

ASAT, Aspartate aminotransferase.
ALAT, Alanine aminotransferase.
For each rat, values are means of triplicate determinations.

Table 2. Liver prolidase and prolinase activity during CCl_4 intoxication

	Number of rats with histological cirrhosis	Prolidase μmol/min/mg mean ± s.e.m	Prolinase μmol/min/mg mean ± s.e.m
Controls + oil ($n=11$)	0	0.15 ± 0.01	0.55 ± 0.02
Day 2 ($n=6$)	0	0.14 ± 0.01	0.59 ± 0.04
Week 3 ($n=8$)	0	0.18 ± 0.01	0.54 ± 0.02
Week 6 ($n=7$)	2	0.26* ± 0.02	0.53 ± 0.03
Week 10 ($n=5$)	4	0.25* ± 0.03	0.56 ± 0.04
Week 12 ($n=5$)	5	0.23* ± 0.01	0.55 ± 0.03

* Value is significantly different from the control value ($P<0.001$).
For each rat, values are means of triplicate determinations.

weeks, while prolinase activity diminished significantly in kidney.

Liver histology

At the 3rd week, centrilobular zonal necrosis was moderate. The reticulin framework was either condensed or collapsed in the centrilobular zones. There was evidence for new collagen and mucopolysaccharide synthesis with fibroblastic proliferation. Portal triads were expanded with increased connective tissue. There was no cirrhosis.

By the 6th week, necrosis was more severe. There were some areas of confluent necrosis. Necrotic hepatocytes were intensely eosinophilic while viable cells had a finely vacuolated cytoplasm due to the presence of small flat droplets. Inflammatory reaction was polymorphic and mild. Liver cell plates were disorganized, with secondary collapse. Fibrosis extended into the lobule and led to the formation of bridging portal triads with terminal hepatic venules. The parenchyma was separated into irregularly shaped islands by thick bands of connective tissue. Some regenerating nodules were seen. Two rats out of seven had defined cirrhosis.

Week 10 and necrosis was mild. Fibrous septa became thicker and tended to form many regenerating nodules. The number of mesenchymal cells increased in connective tissue. Four rats out of five had irregular cirrhosis.

All rats had cirrhosis by the 12th week. Cirrhosis was mostly regular. The number of regenerating nodules per fibrous septum diminished. Septa contained numerous proliferating ductules and venous radicles or arteries. The number of fibroblasts was smaller and necrosis was absent. Any dysplasia was identified by enlargement and hyperchromatism of hepatocyte nuclei. Cirrhosis was present in five rats out of five; it was regular in three and irregular in two.

During experimentation, the control rats given olive oil exhibited no hepatic lesions.

Discussion

In the present work we used the CCl_4 model in the rat to study changes in prolidase and prolinase activity during acute liver necrosis and the fibrotic process.

The study of acute CCl_4 administration in rat has shown that serum prolinase activity was strongly dependent upon liver necrosis. The modifications in serum prolinase activity observed were very similar to those found for aminotransferases. Serum prolidase activity also increased during acute necrosis, but only slightly and more slowly than did aminotransferases. However, the observed decrease in serum prolidase activity was earlier than that of aminotransferases and prolinase. All these results are in full agreement with those reported for human liver (Myara *et al.* 1985). Within the liver, no significant modifications of prolinase and prolidase activity were observed at the time of peak necrosis.

In the rat, chronically intoxicated with CCl_4, liver necrosis is essential to produce irreversible cirrhosis. As the levels of prolidase and prolinase activity in serum were dependent upon liver necrosis, it was difficult to estimate the specimen involvement of these two serum enzyme activities in relation to the fibrotic process. However, unlike prolinase activity, prolidase activity in the liver changed during the course of fibrosis. By week 3, the first stages of the fibrotic process became visible and prolidase activity began to rise. At week 6, reversible fibrosis was plainly established and prolidase activity was at its maximum. Cirrhosis was advanced by weeks 10 and 12; prolidase activity remained elevated. The mechanism explaining the difference in prolidase and prolinase activation is unknown. The increase in intracellular proline observed during the fibrotic process (Ehrinpreiss *et al.* 1980) does not seem to be responsible for the rise in prolidase activity; the latter appears to be inhibited by this aminoacid (Sjostrom 1974).

Liver fibrosis is a morphological diagnosis which in man requires a biopsy. As liver

biopsies cannot be repeated frequently, several serum markers have been proposed as monitors of the fibrotic process. Proline is the easiest to determine but only alcoholic cirrhosis increases proline levels in serum (Mata *et al.* 1975; Kershenobich *et al.* 1981); in all other types of cirrhosis, modifications in proline metabolism are restricted to the liver. Of the enzymes involved in procollagen synthesis, prolyl-hydroxylase is the best documented. However, its use as a marker is limited, because its activity is extremely unstable and is inhibited by certain substances in serum (Rojkind & Perez Tamayo 1983). Determination of immunoreactive protein avoids these drawbacks but, like prolyl-hydroxylase activity (Stein *et al.* 1970), its serum elevation seems to reflect the altered liver function rather than the rate of collagen synthesis (Kuuti-Savalainen *et al.* 1979). Collagenase activity cannot be tested in serum because of the presence of anticollagenase proteins. However, Ikeda *et al.* (1983) proposed that collagenase activity in the granulocytes might reflect the degree of hepatic fibrosis. Finally, the amino-terminal propeptide of type III procollagen, now commercially available as an assay kit, has been suggested as a marker. Although its level was found to be high in patients with other non-fibrotic diseases such as acute-phase necrosis (Rohde *et al.* 1979; Bolarin *et al.* 1984) and hemochromatosis (Colombo *et al.* 1983), this marker might differentiate persistent from active chronic hepatitis (Igarashi *et al.* 1984; Frei *et al.* 1984; Weigand *et al.* 1984). However, some criticisms of this amino-terminal peptide were made by Rojkind (1984) who questioned its predictive value in liver fibrosis. Furthermore, its low serum level necessitates a radioimmunoassay that limits its use in routine practice. On the basis of preliminary human data (Myara *et al.* 1984*b*; 1985) we proposed plasma prolidase activity as a possible tool for monitoring the fibrotic process; it has the added advantage that the colorimetric determination of prolidase is inexpensive and easy to perform. Recent work by Zuyderhoudt *et al.* (1985) and the present study both show plasma prolidase activity does not rise in the early stages of fibrosis but reflects the later stages of cirrhosis. Further information about the mechanisms of prolidase activation might reveal changes in intracellular procollagen degradation during the fibrotic process in the liver.

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