Intracellular Enzymes of Collagen Biosynthesis in Rat Liver as a Function of Age and in Hepatic Injury Induced by Dimethylnitrosamine

CHANGES IN PROLYL HYDROXYLASE, LYSYL HYDROXYLASE, COLLAGEN GALACTOSYLTRANSFERASE AND COLLAGEN GLUCOSYLTRANSFERASE ACTIVITIES

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The relationship between the changes in the four enzyme activities catalysing intracellular post-translational modifications in collagen biosynthesis were studied in rat liver as a function of age and in experimental hepatic injury induced by the administration of dimethylnitrosamine. During aging, relatively large changes were found in prolyl hydroxylase and lysyl hydroxylase activities, whereas only minor changes took place in collagen galactosyltransferase and collagen glucosyltransferase activities. In hepatic injury, the two hydroxylase activities increased earlier and to a larger extent than did the two glycosyltransferase activities, and the largest increase was found in lysyl hydroxylase activity. The data support previous suggestions that changes in the rate of collagen biosynthesis in the liver cannot be explained simply by a change in the number of collagen-producing cells, but regulation of the enzyme activities existed, so that the two hydroxylase activities altered considerably more than did the two collagen glycosyltransferase activities.

The biosynthesis of collagen involves a number of modifications of the initial polypeptide chains after all the information available in mRNA has been translated. Some of these post-translational modifications take place before collagen is secreted from the cells and some of them occur after the secretion of collagen into the extracellular matrix. The intracellular modifications include the hydroxylation of certain prolyl residues to hydroxyprolyl residues, the hydroxylation of certain lysyl residues to hydroxylysyl residues, the glycosylation of some of the hydroxylysyl residues to galactosylhydroxylysyl residues and the glycosylation of some of the galactosylhydroxylysyl residues to glucosylgalactosylhydroxylysyl residues. These reactions are catalysed by four separate enzymes: prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase (for reviews, see Grant & Prockop, 1972*a,b,c*; Bornstein, 1974; Kivirikko & Risteli, 1976; Prockop *et al.*, 1976).

A number of studies have dealt with changes in prolyl hydroxylase activity in animal and human tissues under various experimental and clinical conditions, whereas the other three enzyme activities have been studied only in a few instances (Grant & Prockop, 1972*a,b,c*; Prockop *et al.*, 1976). An attempt to obtain some information about the relationship between changes in the activities of the four enzymes was made by studying them simultaneously during the development of experimental liver injury induced

by carbon tetrachloride (Risteli & Kivirikko, 1974). The results indicated that an increase in all four enzyme activities preceded an increase in the collagen content of the liver. However, the four enzyme activities were not increased to the same extent, indicating that the higher enzyme activities could not be simply explained by an addition in the number of collagen-producing cells having enzyme activity patterns similar to those of the cells initially present in the liver, but that certain enzymes were either synthesized or activated to a greater extent than the others. The hydroxylase activities increased to a greater extent than the glycosyltransferase activities, the largest change being found in lysyl hydroxylase activity and the smallest in galactosyltransferase activity.

In the present study, an attempt was made to examine further the relationship between changes in the four enzyme activities. Liver injury was induced by administration of dimethylnitrosamine, and the enzyme activities were studied during the development of the fibrosis. This condition differs considerably in character from that induced by carbon tetrachloride; for instance, the latter condition is characterized by a marked accumulation of triglycerides in the liver, whereas relatively minor increases take place after the injury induced by dimethylnitrosamine (Madden *et al.*, 1970; Chvapil & Ryan, 1973). In addition, the four enzyme activities were studied in the liver as a function of age, because age-associated alterations in these enzyme activities have previously

been reported (Mussini *et al.*, 1967; Uitto *et al.*, 1969; Spiro & Spiro, 1971; Anttinen *et al.*, 1973), and it thus seemed possible to use such alterations as another model for studying the relationship between changes in the four enzyme activities.

Experimental

Animals and the preparation of liver samples for assays

The experimental animals were female Long-Evans rats. They were fed on a commercial diet (Hankkija Oy, Helsinki, Finland) and allowed free access to water. In the experiment on the effect of age on the enzyme activities, all the rats, in groups of three, were killed on the same day, the ages of each group being then 0, 10, 20, 75, 225 and 420 days respectively. To study the effect of liver injury, three complete experimental series were carried out, the first lasting 3 days, the second 7 days and the third 21 days. The age of the rats at the beginning of each experimental series was 3 months, and each series contained six rats both in the control group and in the liver-injury group.

Hepatic injury was induced by injecting dimethylnitrosamine intraperitoneally on three consecutive days (days 0–2) in doses of 1 μ l (diluted 1:100 with 0.15 M-NaCl)/100 g body wt. In the experiment lasting 21 days, additional injections were given on days 7–9 and 14–16.

The rats were anaesthetized with diethyl ether, their livers were rapidly removed, immediately frozen in liquid N₂ and weighed in the frozen state. The livers were then stored at –70°C until assayed. As previously reported, no changes occurred in the enzyme activities studied compared with the activities in freshly analysed liver samples (Risteli & Kivirikko, 1974).

The livers were homogenized in a Teflon/glass homogenizer as previously reported (Risteli & Kivirikko, 1974), except that the volume of the solution was 14 ml/g of liver and the buffer contained no dithiothreitol, as the addition of this compound to the buffer has been found to have no effect on any of the enzyme activities studied in crude preparations (Rhoads *et al.*, 1967; Myllylä *et al.*, 1976).

Assays of enzyme activities

The incubations in the assays for prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase activities were carried out as in our previous study on liver injury (Risteli & Kivirikko, 1974), with the following modifications. In the assay of prolyl hydroxylase activity the incubation was carried out for 30 min at 30°C and no dithiothreitol was used in the incubation mixture (Risteli *et al.*, 1976). The [¹⁴C]proline-labelled protocollagen substrate was also prepared in a dif-

ferent manner [see the following paper, Risteli *et al.* (1976)]. The [¹⁴C]lysine-labelled protocollagen substrate was likewise prepared differently. Isolated cells obtained from leg tendons of 100 17-day-old chick embryos were incubated with 250 μ Ci of [¹⁴C]lysine as in the preparation of [¹⁴C]proline-labelled protocollagen substrate [see the following paper, Risteli *et al.* (1976)], and the final substrate was divided into portions of 120 000 d.p.m. and stored frozen. In the assay of collagen galactosyltransferase and collagen glucosyltransferase activities the reaction volume was decreased to 100 μ l, retaining the original concentrations of the reactants.

After incubation with prolyl hydroxylase, the reaction was stopped by adding an equal volume of concentrated HCl, and, after hydrolysis at 120°C overnight, the amount of hydroxy[¹⁴C]proline formed was assayed (Juva & Prockop, 1966). The reaction with lysyl hydroxylase was stopped by adding 10 ml of cold acetone, and hydroxy[¹⁴C]lysine was measured (Blumenkrantz & Prockop, 1969; Kivirikko & Prockop, 1972). The [¹⁴C]galactosylhydroxylysine or [¹⁴C]glucosylgalactosylhydroxylysine formed in the reactions with the two collagen glycosyltransferases was assayed as described by Myllylä *et al.* (1975), except that the paper-electrophoresis step of the assay procedure was omitted (see the Results section).

Other assays

The protein content of the liver homogenates and their 15 000 g supernatants was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. For the assay of hydroxyproline, pieces of liver were hydrolysed overnight with 6 M-HCl at 120°C and the amino acid was determined in the hydrolysates (Kivirikko *et al.*, 1967). The hydroxyproline contents were expressed per mg of protein in the samples assayed by the α -amino N method of Rubinstein & Pryce (1959). The triglycerides were assayed as described by Carlsson (1963).

Results

Comments on assays

Conditions for the extraction and assay of the four enzyme activities in the liver were studied previously, and typical standard curves were shown (Risteli & Kivirikko, 1974). The modifications now used in the preparation of [¹⁴C]proline- and [¹⁴C]lysine-labelled protocollagens (see the Experimental section) further increased the linearity in the assay of prolyl hydroxylase and lysyl hydroxylase activities.

In the assay procedure for the collagen galactosyltransferase and collagen glucosyltransferase activities, the products of the enzymic reactions are purified in several steps, including brief ion-exchange chromatography, the last step being paper electrophoresis

(Myllylä *et al.*, 1975). Experiments were performed to find out whether it was necessary to perform the last step in the procedure, for the following reasons: first, because our preliminary data indicated that only a minor fraction of the radioactivity in the paper-electrophoresis strips was present in positions other than the specific products of the reactions; and secondly, because analysis of the radioactivity in several fractions which were summed up introduced some

inaccuracy. The reactions with both transferases were carried out as described in the Experimental section, and after the ion-exchange chromatography step (Myllylä *et al.*, 1975), no paper-electrophoresis was performed, but four identical samples were pooled and chromatographed in an amino acid analyser (JEOL JLC-5AH) by using a program which clearly separated the hydroxylysine glycosides (Askenasi, 1973; Oikarinen *et al.*, 1976). The results indicated that, in the assay of collagen galactosyltransferase activity, over 80% of the radioactivity chromatographed in the positions of the galactosylhydroxylysine standard, and, in the assay of collagen glucosyltransferase activity, over 95% was in the position of the glucosylgalactosylhydroxylysine standard (Fig. 1). Additional experiments performed with samples of injured livers gave an identical distribution of the radioactivity between the two peaks in the assay of products of the galactosyltransferase reaction. On the basis of these experiments, the paper-electrophoresis step of the original assay procedure was omitted.

Effect of age on the four enzyme activities

The four enzyme activities were studied over an age range of 0–420 days. During this time an increase of about 41-fold took place in the weights of the rats and an increase of about 35-fold in the weights of their livers (Table 1). The enzyme activities were expressed in relation to the liver supernatant protein (Fig. 2). The prolyl hydroxylase activity decreased rapidly to about one-third between days 0 and 75 and a further slight decrease was noted thereafter, the value on day 420 being about one-quarter of that on day 0 (Fig. 2a). The lysyl hydroxylase activity showed a slight increase between days 0 and 10, and decreased thereafter to about one-third of the highest value (Fig. 2b). The age curve for lysyl hydroxylase activity resembles that for prolyl hydroxylase activity, except for the slight initial increase.

Fig. 1. Assay of partially purified products of the glycosylation reactions by amino acid analyser

The samples were treated as described in the text and analysed in a JEOL JLC-5AH amino acid analyser. Fractions were collected every 5 min and the radioactivity was measured in a liquid-scintillation counter (Oikarinen *et al.*, 1976). (a) Product of collagen galactosyltransferase reaction; (b) product of collagen glucosyltransferase reaction. The elution position of the major peaks were identical with those of the galactosylhydroxylysine and the glucosylgalactosylhydroxylysine standards. The peak noted between 25 and 35 min when assaying products of the galactosyltransferase reaction was not identified, but the distribution of radioactivity between this peak and the major peak was identical in samples from normal and injured liver.

Table 1. Weights of the rats and their livers and the protein content in the 15000g supernatant of liver homogenates in the experiment on the effect of age

Each group contained three rats. Results are expressed as means ± s.d.

Age (days)	Weight of rat (g)	Weight of liver (g)	Supernatant protein (mg/g of liver)
0	5.7 ± 0.2	0.25 ± 0.02	144.0 ± 3.2
10	15.2 ± 0.4	0.50 ± 0.02	153.0 ± 1.8
20	34.2 ± 1.4	1.62 ± 0.05	162.8 ± 8.7
75	114.7 ± 4.1	4.72 ± 0.09	181.5 ± 7.6
225	188.7 ± 6.6	7.54 ± 0.54	180.0 ± 6.4
420	232.3 ± 7.4	8.75 ± 0.53	187.5 ± 8.5

Fig. 2. Age-related changes in the four enzyme activities in the liver

The values are expressed as radioactivity (d.p.m.) of product formed/mg of protein in the 15000g supernatant of the liver homogenate. Each group contained three rats, and the values are means ($\pm$ S.D., shown by the bars). (a) Prolyl hydroxylase; (b) lysyl hydroxylase; (c) collagen galactosyltransferase; (d) collagen glucosyltransferase activity.

The age-related changes in the two collagen glycosyltransferase activities differed clearly from those in the two hydroxylase activities (Fig. 2c and 2d). Both curves show maximal values on day 10 and lowest values on day 20 or 75. The lowest values for both transferase activities were about three-quarters of their highest values.

Effect of hepatic injury on the four enzyme activities

The changes in the enzyme activities were studied on days 3, 7 and 21 after commencement of dimethylnitrosamine administration. No significant changes were found in the weights of the rats after the treatment (not shown). The wet weight of the livers increased, but this was largely due to an increase in the water content, as there was a significant decrease in the protein content of the livers (Table 2). No change was found in the ratio of the liver supernatant protein

to that of total liver protein. The triglyceride content in the injured livers was about doubled on day 3, but did not differ significantly from that in the controls on day 21. However, even the highest increase in the triglyceride content was low compared with that induced by carbon tetrachloride injections, after which an increase of up to about 13-fold was found (Risteli & Kivirikko, 1974). No change was found in the hydroxyproline content in relation to liver protein on day 3 or 7, whereas an increase of about 20% was found on day 21 (Table 2).

The changes in the four enzyme activities, compared with values in the control rats on each of the time-points, are shown in Fig. 3. On day 3, an increase of about 60% was found in prolyl hydroxylase and lysyl hydroxylase activities, whereas no changes were observed in collagen galactosyltransferase or collagen glucosyltransferase activities. During subsequent days the highest increase was found in the lysyl

Table 2. *Effect of hepatic injury on the weights of the livers, on the protein content in the 15000g supernatants of liver homogenates and on the protein, hydroxyproline and triglyceride contents of the livers*

Each group contained six rats (see the Experimental section). Results are expressed as means $\pm$ s.d., and the significance of the differences was assessed by Student's *t* test. Significance levels: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Time and group	Wt. of liver (as % of wt. of rat)	Supernatant protein (mg/mg of homogenate protein)	Protein content (mg/g of liver)	Hydroxyproline content (μ g/mg of protein)	Triglyceride content (mg/g of liver)
3 days					
Control	3.9 $\pm$ 0.1	0.86 $\pm$ 0.03	210.0 $\pm$ 10.5	1.48 $\pm$ 0.25	4.00 $\pm$ 1.16
Treated	4.2 $\pm$ 0.3***	0.86 $\pm$ 0.02	190.5 $\pm$ 4.1**	1.51 $\pm$ 0.30	8.85 $\pm$ 3.82**
7 days					
Control	4.0 $\pm$ 0.2	0.86 $\pm$ 0.04	209.0 $\pm$ 3.8	1.49 $\pm$ 0.14	4.23 $\pm$ 0.40
Treated	5.2 $\pm$ 0.3***	0.85 $\pm$ 0.02	188.5 $\pm$ 7.1***	1.52 $\pm$ 0.68	5.32 $\pm$ 0.99**
21 days					
Control	4.2 $\pm$ 0.4	0.84 $\pm$ 0.02	220.0 $\pm$ 6.8	1.48 $\pm$ 0.26	4.20 $\pm$ 0.44
Treated	5.0 $\pm$ 0.4**	0.86 $\pm$ 0.03	183.3 $\pm$ 12.2***	1.79 $\pm$ 0.17*	4.73 $\pm$ 0.55

Fig. 3. *Changes in the four enzyme activities in the liver in hepatic injury*

The values are expressed as percentages (means $\pm$ s.d.) of the mean values in the control rats at each of the time-points. Each group contained six rats. The injections of dimethylnitrosamine are indicated by arrows. ■, Prolyl hydroxylase; ●, lysyl hydroxylase; □, collagen galactosyltransferase; ○, collagen glucosyltransferase.

hydroxylase activity. The increase in prolyl hydroxylase activity was clearly less than that in lysyl hydroxylase activity, but clearly more than that in the two collagen glycosyltransferase activities. The latter enzyme activities showed only slight increases during the whole observation period, the highest increase in collagen glucosyltransferase activity being about 26% and in collagen galactosyltransferase activity being about 20% (Fig. 3).

Discussion

Because the biosynthesis of collagen involves a number of post-translational modifications, it seems possible that the rate of biosynthesis of this protein would be partly regulated at the level of some of these modifications. Numerous studies have dealt with changes in prolyl hydroxylase activity in various experimental and clinical states, and in many of such studies it has been suggested that this enzyme activity

may be rate-limiting for collagen biosynthesis (see Grant & Prockop, 1972a,b,c; Cardinale & Udenfriend, 1974; Kivirikko & Risteli, 1976; Prockop *et al.*, 1976). However, no definite proof for this suggestion has been presented.

In the present work an attempt was made to study in the liver the relationship between the changes in the activities of four enzymes that are known to catalyse the intracellular post-translational modifications in collagen biosynthesis. The only previous, related study indicated that considerable differences can be found in the magnitudes of the increases in the four enzyme activities (Risteli & Kivirikko, 1974). The present data further support this finding. Relatively large changes were found in the two hydroxylase activities with age and in experimental liver injury, whereas only minor changes took place in the two collagen glycosyltransferase activities.

Studies have indicated that all four enzymes considered in the present paper are at least mainly located within the cisternae of the endoplasmic reticulum (Olsen *et al.*, 1973; Diegelmann *et al.*, 1973; Harwood *et al.*, 1974, 1975; Cutroneo *et al.*, 1974; Prockop *et al.*, 1976), although some collagen glycosyltransferase activity was also found in the smooth-endoplasmic-reticulum fraction (Harwood *et al.*, 1975). It has been suggested that all these enzymes may reside in a contiguous relationship as a multi-enzyme system bound to the internal face of the cisternae of the rough endoplasmic reticulum (Harwood *et al.*, 1975). In this respect it is noteworthy that relatively large differences are found between the hydroxylase and the glycosyltransferase activities in the conditions studied here. However, it is not known whether the larger changes in the hydroxylase activities were actually due to larger changes in the rate of synthesis of these two enzyme proteins. Comparison of changes in the prolyl hydroxylase activity with those in the amounts of immunoreactive prolyl hydroxylase protein indicated that only relatively minor changes, comparable with those in the two collagen glycosyltransferase activities, took place in the content of this protein in liver with age and in experimental injury [see the following paper, Risteli *et al.* (1976)]. It thus seems possible that part of the changes in the prolyl hydroxylase activity was not due to changes in the content of the enzyme protein. No information is available on the possible presence of an inactive form of lysyl hydroxylase in any tissue, but if prolyl hydroxylase activity was partly controlled without change in the amount of enzyme protein, the parallelism between changes in prolyl hydroxylase and lysyl hydroxylase activities suggests that lysyl hydroxylase activity may be controlled by a similar mechanism.

It is noteworthy that in dermal-scar collagen an increased hydroxylation of lysyl residues is found (Bailey *et al.*, 1975; Shuttleworth *et al.*, 1975), and

that in liver injury the largest increase was found in the lysyl hydroxylase activity. On the other hand, rabbit corneal-scar collagen contains less glucosylated hydroxylysine than does normal corneal collagen (Cintron, 1974), and in liver injury only slight increases were found in the collagen glycosyltransferase activities compared with those in the lysyl hydroxylase activity. It thus seems desirable to study the structure of collagen synthesized in liver fibrosis to find out whether it differs from normal liver collagen with respect to the extent of hydroxylation of lysyl residues and glycosylation of hydroxylysyl residues.

The present data support our previous suggestion that changes in the rate of collagen biosynthesis in the liver cannot be explained simply by a change in the number of collagen-producing cells having similar enzyme activity patterns to those of the cells initially present in the liver (Risteli & Kivirikko, 1974), but regulation of the enzyme activities existed, so that the two hydroxylase activities altered considerably more than did the two collagen glycosyltransferase activities during aging and in experimental liver injury.

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