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Activities of Prolyl Hydroxylase, Lysyl Hydroxylase, Collagen Galactosyltransferase and Collagen Glucosyltransferase in the Liver of Rats with Hepatic Injury

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The activities of four enzymes catalysing post-translational modifications of the collagen polypeptide chains were assayed in the livers of rats with experimental hepatic injury. The liver injury was induced by injecting carbon tetrachloride twice weekly, and assays of the enzymic activities were carried out 2 and 4 weeks after commencement of administration of carbon tetrachloride. The liver homogenates were preincubated with Triton X-100 before the assays, because such treatment was found to increase the activities of all four enzymes in the supernatants of liver homogenates. The activities of all four enzymes had increased by 2 weeks after commencement of carbon tetrachloride administration. No increase was found in the collagen content of the livers at this stage and thus an increase in all four enzyme activities preceded an increase in the collagen content of the liver. A further slight increase was found in three of the enzyme activities during the subsequent 2 weeks of the experiment, whereas no further increase was found in the collagen galactosyltransferase activity. A statistically significant correlation was found between all four enzyme activities, but the magnitude of the increases varied considerably. The largest increase was found in lysyl hydroxylase activity, and at 4 weeks the magnitude of this was about three times that of the collagen galactosyltransferase activity. The results thus indicate that the increased enzyme activities cannot be explained simply by an increase in the number of collagen-producing cells having similar enzyme activity patterns to those of the cells initially present in the liver.

Collagen is the major component of animal connective tissues and the most abundant protein in the body. During the last few years the metabolism of this protein has been the subject of a number of studies and considerable information has been gained on its biosynthesis. This has been found to occur in several relatively discrete stages and one of the special features is that the initial polypeptide chains are modified by several steps after all the information available in messenger RNA has been translated. These steps include the hydroxylation of appropriate prolyl residues to hydroxyprolyl residues, the hydroxylation of appropriate lysyl residues to hydroxylysyl residues, the substitution of some of the hydroxylysyl residues with galactose in *O*-glycoside linkage and substitution of some of the galactosylhydroxylysyl residues with glucose to form glucosylgalactosylhydroxylysyl residues. All these reactions take place before collagen is secreted from the cells into the extracellular matrix, and they are catalysed by four separate enzymes: prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase (for reviews see Grant & Prockop, 1972*a*,*b*,*c*; Kivirikko, 1974; Bornstein, 1974).

Prolyl hydroxylase has been isolated in a highly purified form from two sources (Halme *et al.*, 1970; Rhoads & Udenfriend, 1970; Berg & Prockop, 1973) and there have been numerous reports on changes in the activity of the enzyme in animal and human tissues under various experimental and clinical conditions (Grant & Prockop, 1972*a*,*b*,*c*), whereas relatively little is known about the other three enzymes. Lysyl hydroxylase has been purified 300–600-fold from chick embryo extract (Kivirikko & Prockop, 1972) and has been found to resemble prolyl hydroxylase in several of its properties (Kivirikko & Prockop, 1972; Kivirikko *et al.*, 1972). The activity of lysyl hydroxylase in human skin was highest in foetuses, and higher in infants than in adult subjects (Anttinen *et al.*, 1973). The enzyme activity was markedly decreased in cultured skin fibroblasts from two patients with a deficiency of hydroxylysine in collagen (Krane *et al.*, 1972). In developing chick embryos, changes in the lysyl hydroxylase activities agreed with data on changes in prolyl hydroxylase activity and collagen biosynthesis (Ryhänen & Kivirikko, 1974). No other data are available on changes in lysyl hydroxylase activity. Collagen galactosyltransferase and collagen glucosyltransferase have been purified 15–20-fold from rat kidney cortex, and some initial information is available on the properties of these enzymes (R. G. Spiro & M. J. Spiro, 1971*a*; M. J. Spiro & R. G. Spiro, 1971). Changes in the activities of these enzymes have been reported with age in various tissues, in the uterus during pregnancy and in rat kidney cortex in experimental diabetes (R. G. Spiro & M. J. Spiro, 1971*b*).

Hepatic injury, which leads to fibrosis of the liver, has been reported to be accompanied by an increase in prolyl hydroxylase activity, and this change was found to take place before an increase in the collagen content of the liver (Takeuchi *et al.*, 1967; Takeuchi & Prockop, 1969; Feinman & Lieber, 1972). Since nothing is known about the activities of the other three enzymes in this condition, we decided to study whether the activities of the other enzymes also increased. Further, attention was paid to the questions of whether a possible increase in the activities of these enzymes would precede an increase in the collagen content of the liver and whether the magnitude of the increases would be similar. As the activities of the three enzymes had not been studied previously in the liver tissue, the conditions for assaying these enzymes in the liver were also examined.

Experimental

Experimental animals and induction of hepatic injury

The experimental animals were female Long-Evans rats, aged 4 months at the beginning of the experiments. They were fed on a commercial diet (Hankkija Oy) and allowed free access to water. After preliminary experiments, two complete experimental series were carried out, the first lasting 2 weeks and the second lasting 4 weeks. In both experiments the control group and the liver-injury group both contained six rats. Their weights and the weights of their livers are indicated in Table 2 of the Results section.

Hepatic injury was induced by injecting carbon tetrachloride subcutaneously twice weekly in doses of 0.05ml per 100g of body weight, diluted with an equal volume of mineral oil. Thus the rats in the 2-week experiment received four injections and those in the 4-week experiment received eight.

Preparation of liver samples for assays

Rats were anaesthetized with ether 3 days after the last injection of carbon tetrachloride and their livers were rapidly removed and immediately frozen in liquid nitrogen. The livers were then stored at −20°C until assayed. Control experiments indicated that no changes occurred in the activities of the enzymes studied compared with activities in freshly analysed liver samples.

The livers were homogenized in a Teflon and glass homogenizer (Thomas) at about 1500rev./min for 60s in a cold (0°C) solution consisting of 0.2M-NaCl, 0.1M-glycine, 50μM-dithiothreitol, 0.1% (w/v) Triton X-100 and 20mM-Tris–HCl buffer, adjusted to pH7.5 at 4°C. The volume of the solution was 9ml/g of liver. The homogenates were incubated at 4°C for 60min and then centrifuged at 15000*g* for 30min at 4°C. Portions of the supernatant were then used for assay of the enzyme activities and of the supernatant protein. Separate pieces of the liver were used for assay of total protein, hydroxyproline and triglycerides as indicated below.

Assay of prolyl hydroxylase activity

Portions of the 15000*g* supernatants were incubated with agitation for 45min at 37°C in a final volume of 2ml containing 50000d.p.m. of [^{14}C]proline-labelled protocollagen substrate, 0.08mM-$FeSO_4$, 2mM-ascorbic acid, 0.5mM-α-oxoglutarate, 0.2mg of catalase (Calbiochem Ltd., London W.1, U.K.)/ml, 0.1mM-dithiothreitol (Calbiochem), 2mg of bovine serum albumin (Sigma Chemical Co., Kingston-upon-Thames, U.K.)/ml, and 50mM-Tris–HCl buffer, adjusted to pH7.8 at 25°C (see Halme *et al.*, 1970). The reaction was stopped by adding an equal volume of concentrated HCl, and after hydrolysis at 120°C overnight, the amount of hydroxy[^{14}C]proline formed was assayed (Juva & Prockop, 1966). The [^{14}C]proline-labelled protocollagen substrate was prepared as described previously (Kivirikko & Prockop, 1967) and boiled for 5min (Takeuchi *et al.*, 1967) before being divided for storage into portions of 50000d.p.m.

Assay of lysyl hydroxylase activity

Portions of the 15000*g* supernatants were incubated with agitation for 60min at 37°C in a final volume of 1ml containing 200000d.p.m. of [^{14}C]lysine-labelled protocollagen substrate, 0.05mM-$FeSO_4$, 0.5mM-ascorbic acid, 0.5mM-α-oxoglutarate, 0.1mg of catalase, 0.1mM-dithiothreitol, 1.5mg of bovine serum albumin and 50mM-Tris–HCl buffer, adjusted to pH7.8 at 25°C (Kivirikko & Prockop, 1972). The enzymic reaction was stopped by adding 10ml of cold acetone, and hydroxy[^{14}C]lysine in the samples was assayed as described previously (Blumenkrantz & Prockop, 1969; Kivirikko & Prockop, 1972).

Assay of collagen galactosyltransferase and collagen glucosyltransferase activities

In order to assay the galactosyltransferase activity, portions of the 15000*g* supernatant were incubated with shaking for 60min at 37°C in a final volume of 200μl containing 40mg/ml of gelatinized collagen substrate, 60μM-UDP-[^{14}C]galactose [New England Nuclear Corp. (Boston, Mass., U.S.A.), diluted with the unlabelled compound (Sigma) to a final specific radioactivity of 55Ci/mol], 10mM-$MnCl_2$ and 50mM-Tris–HCl buffer, adjusted to pH7.4 at 20°C (R. Myllylä, L. Risteli & K. I. Kivirikko, unpublished work). The assays of glucosyltransferase activity were carried out in 200μl of a similar mixture, except that 60μM-UDP-[^{14}C]glucose [New England Nuclear, diluted with the unlabelled compound (Sigma) to a

final specific radioactivity of 12Ci/mol] was used (R. Myllylä, L. Risteli & K. I. Kivirikko, unpublished work).

Both enzymic reactions were stopped by adding 2ml of cold 1% (w/v) phosphotungstic acid in 0.5M-HCl (Kirschbaum & Bosmann, 1973), and after centrifugation at about 4000*g* for 10min the pellets were washed twice with 10% (w/v) trichloroacetic acid and once with ethanol–ether (1:1, v/v). The dried pellets were hydrolysed with 1.5ml of 2M-NaOH at 105°C for 18h and the [^{14}C]galactosylhydroxylysine or [^{14}C]glucosylgalactosylhydroxylysine formed was assayed by a recently devised specific procedure (R. Myllylä, L. Risteli & K. I. Kivirikko, unpublished work) involving further purification of the samples in small columns containing Dowex 50-X8, and paper electrophoresis in pyridine–acetate buffer, pH3.5.

Other assays

The protein content of the 15000*g* supernatant was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. The total protein of the liver samples was assayed after hydrolysis in 6M-HCl at 120°C overnight using the α-amino nitrogen method of Rubinstein & Pryce (1959). The hydroxyproline content of the liver samples was assayed in samples of the same hydrolysates by the method of Kivirikko *et al.* (1967). The triglycerides were assayed as described by Carlsson (1963).

All ^{14}C radioactivity counting was performed in a Wallac liquid-scintillation spectrometer with an efficiency of 85% and a background of 25c.p.m.

Results

Conditions for the assay of enzyme activities in the liver

A recent study indicated that the addition of detergents to liver homogenates prepared in 0.25M-sucrose by a mild homogenization technique resulted in a 2–3-fold increase in the prolyl hydroxylase activity extracted (Guzman & Cutroneo, 1973). An increase of 30–40% was also found in the lysyl hydroxylase activity of chick embryo extracts after preincubation of the homogenates with Triton X-100 (Ryhänen & Kivirikko, 1974). Accordingly, the effect of Triton X-100 on the activities of the four enzymes in the supernatants of the liver was tested under the present enzyme extraction conditions. Preincubation of the liver homogenates with 0.1% (w/v) Triton X-100 for 1h was found to increase the activity of all four enzymes (Table 1), and thus all experiments reported below were carried out with extracts of the homogenates treated with Triton X-100.

To ensure that assays of all enzyme activities were carried out under conditions in which the relationship between enzyme activity and product formation was linear, standard curves were prepared for all enzymes with each batch of the biologically prepared substrate. Typical standard curves for the reactions are shown in Fig. 1. The standard curves obtained for prolyl and lysyl hydroxylase activities deviated from linearity, especially with a large amount of the liver extract, so that it was necessary to carry out the assays of prolyl hydroxylase activity with samples of only 0.015 or 0.020ml of liver supernatant and the assays of lysyl hydroxylase activity with 0.02 or 0.03ml samples. The standard curves for the collagen glycosyltransferase activities were more linear and assays of both transferase activities were carried out with 0.04ml samples.

Effect of hepatic injury on the weights of the rats and their livers, and on the protein, hydroxyproline and triglyceride contents of the livers

Hepatic injury had no effect on the weights of the rats, but the weights of the livers were about 25%

Table 1. *Effect of Triton X-100 on the activities of prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glycosyltransferase in the 15000g supernatants of rat liver homogenates*

Five livers were divided into two pieces each. One piece of each liver was homogenized as described in the Experimental section. The other piece was homogenized identically, except that the homogenization buffer did not contain Triton X-100. The enzymic activities were determined in the 15000*g* supernatant of the liver homogenates as described in the Experimental section.

Enzymic activity	Addition of Triton X-100	Product formed (d.p.m./mg of liver, mean±S.D.)	% of value obtained without Triton X-100
Prolyl hydroxylase	−	416±25	
	+	509±68	122*
Lysyl hydroxylase	−	126±12	
	+	177±24	140†
Galactosyltransferase	−	99±31	
	+	164±28	166*
Glucosyltransferase	−	205±49	
	+	311±48	152*

* $P<0.01$.
† $P<0.001$.

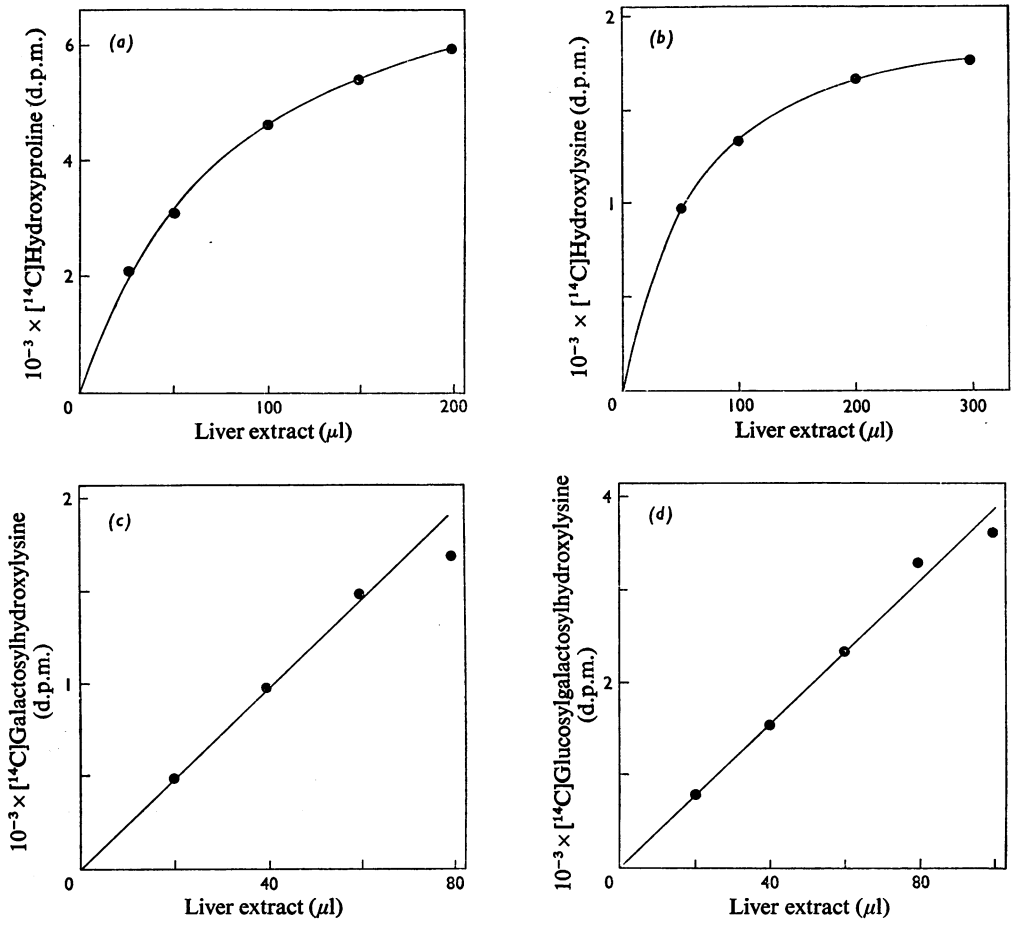

Fig. 1. *Effect of enzyme concentration on product formation in the enzymic reactions*

The enzyme concentration is expressed as μl of the 15000*g* supernatant of the liver homogenate in the standard incubation volume for each enzymic reaction. (*a*) Prolyl hydroxylase; (*b*) lysyl hydroxylase; (*c*) collagen galactosyltransferase; (*d*) collagen glucosyltransferase.

Table 2. *Effect of hepatic injury on the weights of the rats and their livers, and on the protein, hydroxyproline and triglyceride contents of the livers*

Each group contained six rats. Determinations were carried out 2 and 4 weeks after commencement of administration of carbon tetrachloride. Results are expressed as mean±S.D.

Time and group	Weight of rat (g)	Weight of liver (g)	Protein content (mg/g of liver)	Hydroxyproline content (μg/mg of protein)	Triglyceride content (mg/g of liver)
2 weeks					
Controls	203±11	7.1±0.3	233±11	1.12±0.27	4.6±0.8
Treated	206± 8	9.0±0.9†	205±19*	1.10±0.03	48.5±7.5†
4 weeks					
Controls	206±23	7.8±1.0	232±12	1.02±0.17	4.3± 0.6
Treated	200±14	9.6±0.6*	187±17†	1.48±0.27*	54.0±25.6†

* $P<0.01$.
† $P<0.001$.

Table 3. *Effect of hepatic injury on protein content and prolyl hydroxylase and lysyl hydroxylase activities in the 15000g supernatant of liver homogenates*

Each group contained six rats. Determinations were carried out 2 and 4 weeks after commencement of administration of carbon tetrachloride. Results are expressed as mean±S.D. Supernatant protein is expressed in mg/mg of total protein in the liver. The enzyme assays at 2 and 4 weeks were carried out in separate incubations and their values are only comparable within the same experiment.

Time and group	Supernatant protein (mg/mg of total protein)	Prolyl hydroxylase activity		Lysyl hydroxylase activity	
		d.p.m./mg of supernatant protein	Change (%)	d.p.m./mg of supernatant protein	Change (%)
2 weeks					
Controls	0.82±0.05	4540± 220		1120±110	
Treated	0.75±0.11	8530±1580*	+88	2230±440*	+99
4 weeks					
Controls	0.76±0.05	5620± 450		810± 90	
Treated	0.78±0.07	12090±2230*	+115	1950±560*	+141

* $P<0.001$.

Table 4. *Effect of hepatic injury on collagen galactosyltransferase and collagen glucosyltransferase activities in the 15000g supernatant of liver homogenates*

Determinations were carried out 2 and 4 weeks after commencement of administration of carbon tetrachloride. Results are expressed as mean±S.D. The enzyme assays at 2 and 4 weeks were carried out in separate incubations and their values are only comparable within the same experiment.

Time and group	Galactosyltransferase activity		Glucosyltransferase activity	
	d.p.m./mg of supernatant protein	Change (%)	d.p.m./mg of supernatant protein	Change (%)
2 weeks				
Controls	700±150		2330±250	
Treated	990±260*	+41	3600±730†	+55
4 weeks				
Controls	960±160		2290±760	
Treated	1380±300†	+44	3950±590‡	+73

* $P<0.05$.
† $P<0.01$.
‡ $P<0.001$.

higher in rats with hepatic injury (Table 2). The protein content of the liver was 12–19% lower in rats with hepatic injury, and thus there was no significant change in the total amount of protein. The hydroxyproline assays indicated that there was no change in the collagen content of the livers 2 weeks after the hepatic injury when this content was expressed per mg of liver protein. However, about a 45% increase in the hydroxyproline values was found 4 weeks after injury (Table 2). A marked increase was found in the triglyceride content of the livers both 2 weeks and 4 weeks after injury (Table 2).

Changes in liver prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase, and collagen glucosyltransferase activities after hepatic injury

Increased activity of all four enzymes was found in the liver 2 weeks after the commencement of the administration of carbon tetrachloride (Tables 3 and 4). The increase in prolyl and lysyl hydroxylase activity was more marked than that in the activities of the collagen glycosyltransferases. As indicated above, there was no increase in the collagen content of the livers at this stage of the experiment (Table 2), and thus the increase in the activities of all four enzymes could be said to have preceded any increase in the collagen content.

A further slight increase was found with three enzyme activities during the subsequent 2 weeks of the experiment, but the collagen galactosyltransferase activity remained unchanged (Tables 3 and 4). At this stage of the experiment the largest increase was found in the lysyl hydroxylase activity and the smallest increase in the collagen galactosyltransferase activity. The increase in the former enzyme activity was about 3 times that in the latter.

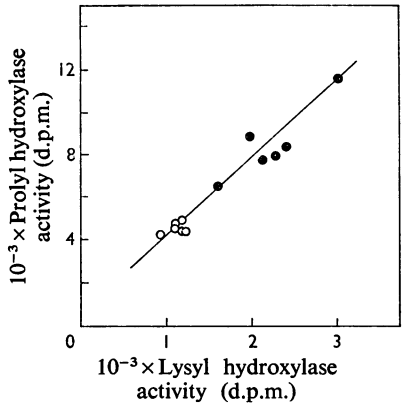

Fig. 2. *Correlation between prolyl hydroxylase and lysyl hydroxylase activities in the livers of rats 2 weeks after commencement of administration of carbon tetrachloride*

Both enzymic activities were expressed as 10^{-3} × d.p.m. of the product formed under standard assay conditions. ○, Controls; ●, carbon tetrachloride-treated rats.

The correlations between the following enzyme activities were tested 2 weeks after the beginning of the experiment: lysyl hydroxylase versus prolyl hydroxylase ($r = 0.968$, $P < 0.001$); lysyl hydroxylase versus collagen glucosyltransferase ($r = 0.874$, $P < 0.001$); lysyl hydroxylase versus collagen galactosyltransferase ($r = 0.679$, $P < 0.01$) and collagen galactosyltransferase versus collagen glucosyltransferase ($r = 0.695$, $P < 0.01$). Thus it is seen that there were statistically significant correlations between the values for all four enzyme activities. The correlation between the prolyl and lysyl hydroxylase activities is shown in Fig. 2.

Discussion

The biosynthesis of collagen fibres is a complex process, and it involves a number of enzymic reactions which occur after all the information in the mRNA has been translated (Grant & Prockop, 1972*a*,*b*,*c*; Kivirikko, 1974; Bornstein, 1974). Several studies have indicated that under certain conditions relatively large changes can take place in the enzyme activities catalysing these reactions. Most of these reports deal with changes in the activity of prolyl hydroxylase, which has been found to increase in a number of experimental and clinical conditions, accompanied by an increased rate of collagen biosynthesis (see Grant & Prockop, 1972*a*,*b*,*c*; Kivirikko, 1974; Bornstein, 1974). Of special interest is the finding that prolyl hydroxylase is present in fibroblasts in culture partly as an inactive proenzyme and that the addition of the ascorbate or lactate to the cultures activates the inactive enzyme precursor (McGee *et al.*, 1971; Stassen *et al.*, 1973; Levene *et al.*, 1974). Recent studies suggest that the proenzyme is also present in mammalian tissues, and that the activation of prolyl hydroxylase may thus prove to be of physiological significance (Stassen *et al.*, 1974).

Unlike changes in prolyl hydroxylase activity, changes in lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase activities have been studied only in a few instances. There are no data to indicate whether these three enzymes are in the tissues partly in the form of proenzymes and whether activation of these would have any physiological significance.

In the present study an attempt was made to gain some information about the relationship between changes in the activities of the four enzymes in experimental fibrosis of the liver. This condition seemed to be suitable for such a study because the initial collagen content of the liver is low, and a rapid and marked increase in the content of this protein is known to take place during experimental fibrosis. Further, previous studies indicated that prolyl hydroxylase activity did increase in the presence of this condition, and that this increase occurred at an early stage in the fibrosis, preceding any increase in the collagen content of the liver (Takeuchi *et al.*, 1967; Takeuchi & Prockop, 1969; Feinman & Lieber, 1972).

In order to be able to compare the magnitudes of possible changes in the activities of the enzymes, it was necessary to ascertain whether the assays were carried out under optimal conditions. With the final procedures used here, the relationship between product formation and enzyme activity was linear for collagen galactosyltransferase and collagen glucosyltransferase, whereas a deviation from the linearity was noted with prolyl and lysyl hydroxylases. However, with the liver-extract samples used, the deviation from linearity could be decreased to a minimum. Another critical factor was the optimal extraction of the enzymes from the liver. Preincubation of the homogenates with Triton X-100 was found to cause a slight increase in the activities of all four enzymes, and thus such a step was incorporated in the assay procedures.

The activities of all four enzymes were found to have increased by 2 weeks after commencement of carbon tetrachloride injections. During this stage of the experiment there was no increase in the collagen content of the liver, and thus an increase in all four enzyme activities preceded any increase in the collagen content. Statistically significant correlations

were found between the activities of the four enzymes, but the magnitudes of the increases varied considerably. The largest increase was found in lysyl hydroxylase activity, which was at 4 weeks about three times that noted in collagen galactosyltransferase activity.

The mechanisms involved in the increases in these enzyme activities and in the production of the excess of collagen during hepatic fibrosis are not known in detail. Some studies suggest that essentially all prolyl hydroxylase activity in the liver is present in the parenchymal cells (Ohuchi & Tsurufuji, 1972), whereas others suggest that liver mesenchymal cells are responsible for the prolyl hydroxylase activity (Shaba *et al.*, 1973) and for collagen production (Popper & Udenfriend, 1970; McGee & Patrick, 1972; Shaba *et al.*, 1973). A recent study clearly indicated that cloned liver parenchymal cells in culture show prolyl hydroxylase activity (Langness & Udenfriend, 1974), but the results do not solve the problem of whether these cells synthesize collagen in liver fibrosis.

Our present data do not indicate which cells are responsible for collagen production in liver fibrosis, but there is an indication that the increased enzyme activities cannot simply be explained by an increase in the number of collagen-producing cells having enzyme activity patterns similar to those of the cells initially present in the liver. The magnitudes of the increases differed considerably, suggesting that certain enzymes were either synthesized or activated to a greater extent than others. As discussed above, prolyl hydroxylase is known to be present in the tissues partly as an inactive proenzyme, and thus at least part of the increase in the activity of this enzyme may be due to the activation of the proenzyme. In this respect it is of interest that in the present study the largest increase was found in lysyl hydroxylase activity. It remains to be studied whether this enzyme also is present in tissues as an inactive proenzyme.

Although a number of studies have been carried out on the enzymes of collagen biosynthesis, there are no definite data to indicate whether some of them are rate-limiting enzymes of the biosynthetic pathway. The present results show that the collagen glycosyltransferase activities were increased by liver fibrosis to a lesser extent than the hydroxylase activities, and thus it seems that a smaller increase is required in the glycosyltransferase activities than in the hydroxylase activities for increased collagen synthesis in this condition.

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