INCREASED PROTOCOLLAGEN HYDROXYLASE ACTIVITY IN THE LIVERS OF RATS WITH HEPATIC FIBROSIS*

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Recent studies have demonstrated that the hydroxyproline and hydroxylysine in collagen are synthesized by the hydroxylation of proline and lysine in a large polypeptide precursor of collagen called protocollagen (for review see 1). The soluble enzyme protocollagen hydroxylase (2) hydroxylates the proline (1,2,3) and probably the lysine (2) in protocollagen polypeptides with a molecular weight of about 100,000 (4,5), and both hydroxylations require atmospheric oxygen, ascorbate, ferrous iron, and α-ketoglutarate (see 1,2,3). The purified enzyme does not hydroxylate free proline or proline in tripeptides, but it hydroxylates proline in synthetic polytripeptides such as $(gly\text{-}pro\text{-}pro)_n$ (6,7) and $(gly\text{-}ala\text{-}pro)_n$**. The hydroxylation of protocollagen is one of the terminal reactions in the intracellular synthesis of collagen, and therefore the level of protocollagen hydroxylase activity in tissues may be an indication of the rate of collagen fiber formation. In the present report the activity of protocollagen hydroxylase was examined in livers of rats with hepatic fibrosis.

Male Sprague-Dawley rats with an average weight of 150 g were divided into two groups. The experimental group received 0.05 ml per 100 g body weight carbon tetrachloride in an equal volume of mineral oil subcutaneously twice weekly for 2 or 12 weeks. The rats were killed 3 days after the last injection, the livers were removed, and they were quickly frozen for storage. The frozen livers were thawed, and 300 mg pieces were homogenized in 5.7 ml cold 0.05 M KCl

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and 0.01 M Tris-HCl buffer, pH 7.6, in a Teflon and glass homogenizer at about 1,500 rpm for 90 seconds. The homogenate was centrifuged at 15,000 x g at 4° for 30 minutes. In order to assay protocollagen hydroxylase activity, 0.2 ml of 15,000 x g supernate corresponding to the supernate from 10 mg whole liver was incubated for 60 minutes at 37° with 50,000 dpm proline-labeled protocollagen-^{14}C substrate; 50 mM Tris-HCl buffer, pH 7.8 at 25°; 2 mM ascorbic acid; 0.5 mM α-ketoglutarate; 0.05 mM $FeSO_4$; and 0.05 mg per ml catalase (Calbiochem) in a final volume of 8 ml (2,7). After the incubation, 8 ml concentrated HCl was added, and the samples were hydrolyzed overnight at 120°. Total ^{14}C and hydroxyproline-^{14}C (8) were then assayed in the hydrolysates. The proline-labeled protocollagen-^{14}C used as a substrate for the hydroxylase assay was prepared as described previously (2), except that the 100,000 x g supernate was boiled for 5 minutes after dialysis and before it was divided into aliquots of 50,000 dpm for storage. Another aliquot of the 15,000 x g supernate of the liver homogenate was hydrolyzed in 6 N HCl at 120° overnight, and assayed for α-amino nitrogen (9). Also, a separate piece of each liver was hydrolyzed directly in 6 N HCl, and assayed for α-amino nitrogen and hydroxyproline (10).

Table I. Collagen hydroxyproline and protocollagen hydroxylase activity in livers of control rats and rats treated with carbon tetrachloride.[1]

Group	No. of Rats	Protein[2] Total mg/100 mg	Protein[2] Supernate mg/100 mg	Total hypro[3] µg/mg protein	Protocollagen hydroxylase[4] dpm
Expt. 1					
Control	7	13.2±2.3	10.4±1.9	1.6±0.3	1280±360
CCl_4-treated, 12 wk	10	9.7±1.3**	7.2±0.6**	6.0±2.4**	6540±1170***
Expt. 2					
Control	4	13.3±0.5	9.3±1.2	1.6±0.3	2030±280
CCl_4-treated, 2 wk	4	13.6±0.3	9.2±0.6	1.9±0.2	2890±380*

[1] Mean values and standard deviations.
[2] Values expressed per 100 mg wet weight of liver.
[3] Values expressed per mg protein of liver.
[4] Hydroxyproline-^{14}C synthesized from 50,000 dpm proline-labeled protocollagen-^{14}C per mg protein in the 15,000 x g supernate of liver homogenates.
* $p < 0.02$; ** $p < 0.01$; *** $p < 0.001$.

With the assay procedure used here, the activity of protocollagen hydroxylase was readily measured with the 15,000 x g supernate which was obtained from 2 to 10 mg wet weight liver and which contained 0.2 to 1.0 mg protein. When increasing amounts of liver were assayed, the amount of hydroxyproline-^{14}C synthesized approached a limiting value of about 4,000 dpm, probably because inhibitory substances are present in the liver (Fig. 1). Similar evidence for the presence of inhibitory substances in crude extracts of chick embryos has been observed previously (7). With 5 mg (Fig. 2) or 10 mg (not shown) of liver, the synthesis of hydroxyproline-^{14}C increased with the amount of purified enzyme added, indicating that with these amounts of liver the assay can be used to measure increases in enzyme activity over a significant range.

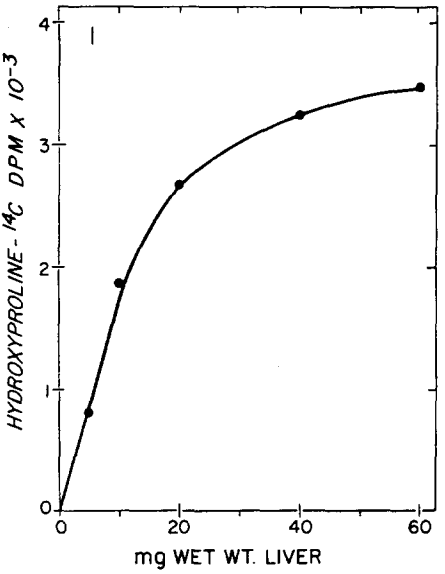

Fig. 1: Activity of protocollagen hydroxylase in normal rat liver. Values indicate dpm hydroxyproline-^{14}C synthesized with 50,000 dpm proline-labeled protocollagen-^{14}C as substrate and varying amounts of the 15,000 x g supernate of a liver homogenate as source of enzyme.

In the first experiment test rats received carbon tetrachloride for 12 weeks. The protein content of the total liver homogenates and of the 15,000 x g supernate of the homogenates was decreased to about 70% of the control value on the basis of wet weight of liver. The collagen hydroxyproline

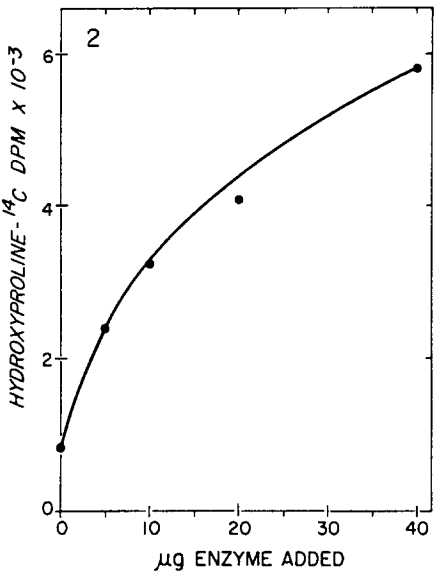

Fig. 2: Assay of purified protocollagen hydroxylase in the presence of rat liver homogenate. Varying amounts of protocollagen hydroxylase purified through the calcium phosphate gel step (2,7) were added to the 15,000 x g supernate from 5 mg wet weight liver. Enzyme activity was assayed as in Fig. 1 and as described in text.

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per mg total liver protein was increased about 3.5-fold. The observed level of protocollagen hydroxylase activity in the livers was increased about 5-fold compared to control values, but the non-linearity of the assay system (Fig. 2) suggests that the true increase of enzymatic activity was even greater.

The livers from rats which received carbon tetrachloride for 2 weeks showed no change in the protein content on the basis of wet weight. There was a slight increase in the mean value for hydroxyproline per mg protein, but the increase was not statistically significant. The observed level of protocollagen hydroxylase activity was significantly increased to about 140% of the control level.

The results presented here as well as those recently reported in healing wounds, scurvy, and old animals (11) suggest that the level of protocollagen hydroxylase activity does reflect the rate of collagen fiber formation in a tissue. With the present procedure the enzyme assay can be performed on as

little as 2 mg wet weight liver, and the results suggest that the increase in the level of protocollagen hydroxylase activity may occur earlier than the increase in hydroxyproline content which reflects the synthesis of new collagen fibers in the liver.

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