References and Notes

1. Mention of a trademark name or a proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.
2. Abbreviations: DDT: 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; DDD: 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane; DDE: 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene TDE: 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane; BHC: 1,2,3,4,5,6-hexachlorocyclohexane; DC-200: silicone, Dow-Corning 200; SE-30: silicone gum rubber (methyl); and QF-1: silicone(fluoro) (FS1265).
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15 June 1967

Collagen Proline Hydroxylase in Wound Healing, Granuloma Formation, Scurvy, and Growth

Abstract. *Compared to homolgous tissues from adult animals, rapidly growing embryonic and fetal tissues contain large amounts of collagen proline hydroxylase. Lung and skin from scorbutic guinea pigs contain one-third as much enzyme as normal tissues do. A rapid increase in proline hydroxylase occurs on the 2nd day of formation of granuloma after injection of carrageenan and on the 4th day of wound healing. The increase in enzyme activity is associated with the onset of collagen biosynthesis and deposition.*

Healing of wounds, formation of granulomas, growth, and differentiation are all characterized by distinctive rates of synthesis of connective tissue, and collagen is the major component of connective tissue in animals. The conversion of prolyl to hydroxyprolyl residues is an enzymatic step uniquely important to collagen biosynthesis, since collagen is the only animal protein that contains hydroxyproline. In a study of the factors that govern the rate of collagen synthesis in intact animals, we determined the amount and specific activity of collagen proline hydroxylase (*1–3*) in different organs and tissues under various experimental conditions. Our data suggest that changes in the amount of this enzyme may be of decisive importance in regulating the rate of formation and deposition of collagen.

Tissues were excised, blotted, weighed, and cut into small pieces with scissors. Ten milliliters of cold 0.25*M* sucrose were added per gram of tissue, and the mixture was homogenized for 1 minute at high speed in a Servall Omni-Mixer. After centrifugation at 15,000*g*, supernatant fractions were collected. Rehomogenization and reextraction of the 15,000*g* pellets yielded approximately 20 percent as much enzyme as the initial extraction. Concentrations of protein were estimated by the method of Lowry (*4*). Collagen proline hydroxylase was assayed in a 2.0-ml volume of incubation mixture by measuring the release of tritium from specially prepared protein substrate labeled with 3,4-^{3}H-proline, as described previously (*2*). Ascorbic acid, α-ketoglutarate, and ferrous ion, cofactors of the enzyme (*2*), were present in optimum amounts. Specific activities of homogenates are reported as counts per minute of hydroxyproline formed per milligram of protein per hour. The standard error of the mean (S.E.) is reported in many cases. The amount of hydroxyproline formed per 30-minute incubation was linearly related to the amount of homogenate added to the assay from 0 to 1000 μg of protein. A requirement for α-ketoglutarate is characteristic of partially purified preparations of peptidyl proline hydroxylase obtained from chick embryo (*2*, *3*), granuloma of guinea pig (*2*), and skin of fetal rat (see *2*).

To rule out the possibility of nonenzymatic release of tritium, crude homogenates were assayed for peptidyl proline hydroxylase activity before and after dialysis, in both the presence and absence of added α-ketoglutarate. Dialyzed extracts were always inactive in the absence of added α-ketoglutarate, whereas the activity of undialyzed extracts was stimulated two- to threefold by its addition. No evidence of nonenzymatic hydroxylation was obtained.

Peptidyl proline hydroxylase activity of homogenates prepared from organs of 2- to 3-month-old Sprague-Dawley rats, weighing 300 g, are shown in Table 1. The enzyme is present in many tissues but is most highly concentrated in extracts of lung, heart, and skin. No activity was detected in whole blood or its components. In general we found that rapidly growing embryonic and fetal tissues contain more proline hydroxylase per unit weight and per milligram of extractable protein than comparable tissues from adult animals do. As shown in Fig. 1, activity in rat tissues was very high in 3-day-old animals, but by 5 to 10 days after birth the specific activity of homogenates decreased to levels comparable to those of adult animals. A characteristic amount of protein was extracted per gram of tissue for each organ (Table 1), and this amount did not vary significantly with changes in the age of the animal.

Chick embryo is convenient for studying variations in the level of peptidyl proline hydroxylase during development. No enzyme activity was detected in either the yolk or the white of the egg; this activity was first detected in 4-day-old embryos weighing 50 to 60 mg. Prior to day 4 it is difficult to obtain sufficient tissue to assay; after day 4 both specific activity and total amount of enzyme in the embryo increased at a rapid rate. On day 6 the average specific activity of homogenates was 500 count/min of hydroxyproline per milligram of protein per hour, whereas on day 14 the average specific activity was 2000. Other investigators (*5*) determined the amount

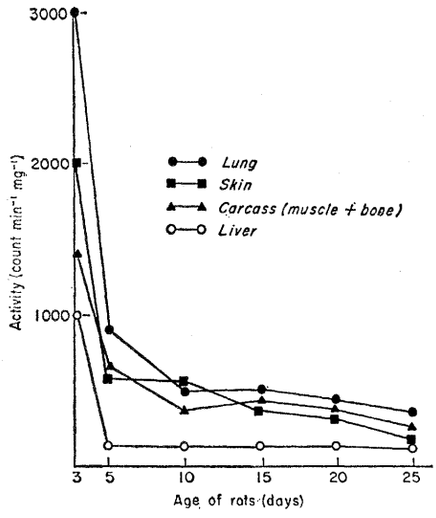

Fig. 1. Changes in the activity of collagen proline hydroxylase in homogenates of tissues from rats of various ages (in counts per minute per milligram of protein). Each point is the average of two determinations on pooled homogenates of four to eight rats. For a single point the actual values obtained never deviated more than 15 percent from the mean.

of hydroxyproline in chick embryos of various ages and found that it first appears on the 5th day of incubation, which is 1 day after we first detected the hydroxylase.

In guinea pigs the formation of hydroxyproline can be modified by dietary restriction of ascorbic acid (*6*). The effects of a deficiency in ascorbic acid on peptidyl proline hydroxylase were investigated. One group of six guinea pigs was kept on a diet free of ascorbic acid; a second group was fed a normal diet supplemented with cabbage. When the animals were killed on day 14, the scorbutic ones had developed intramuscular hematomas, but they were not debilitated. Average activities of peptidyl proline hydroxylase in homogenates of normal lung and skin were 1200 and 240 count/min, respectively; whereas, activities in homogenates of lung and skin from scorbutic animals were 480 and 60 count/min, respectively. In the assay all cofactors including ascorbate were added in excess so that the rate of formation of hydroxyproline depended only on the total amount of enzyme present. There was no difference between scorbutic and normal animals in the amount of protein extracted per gram of tissue homogenized. In guinea pigs ascorbic acid may function as a cofactor for proline hydroxylation and in the maintenance of levels of the enzyme in the tissue. These observations may explain some of the defects in the synthesis of connective tissue that are observed in scurvy (*7*).

Changes in the level of peptidyl proline hydroxylase during the healing of wounds were studied in male Sprague-Dawley rats, weighing 250 g. Under aseptic conditions a cut 5 cm long was made through the skin lateral to the spine (see Fig. 2), and edges of the wound were approximated with stainless steel clips. At various times after wounding, rats were killed and skin from three different areas was removed, homogenized, and assayed for collagen proline hydroxylase. Areas chosen for assay were the wound itself (Fig. 2, circles), strips of skin 3 to 6 mm away from the wound (Fig. 2, triangles), and skin from a normal area contralateral to the wound (Fig. 2, squares). For normal skin an average of 17 mg of soluble protein was obtained per gram of tissue homogenized; for wounds 2 to 14 days old, 30 to 33 mg of protein were obtained. For 3 days enzymatic activity in all areas remained within the normal range; between the 3rd and 4th days an abrupt rise in activity occurred in the wound and continued until day 5, reaching specific activities that were five times the normal. After day 5, hydroxylase activity slowly decreased and reached the normal range 2 to 3 weeks after wounding. A much smaller increase was observed simultaneously in skin a few millimeters away from the wound (Fig. 2), but this returned to control levels within 4 days. The increase in peptidyl proline hydroxylase activity was localized to the vicinity of the wound. The time course observed in these experiments was re-

Table 1. Collagen proline hydroxylase levels in organs of Sprague-Dawley rats. Protein in the extracts is reported as milligrams of protein in the 15,000*g* supernatant per gram of tissue homogenized; hydroxylase specific activity is given as counts per minute (cpm) of hydroxyproline formed per milligram of protein per hour ± S.E.; total enzyme is the product of protein in the extract times specific activity of hydroxylase. Sufficient protein was added to each incubation to form at least 200 count/min of tritiated water and an equivalent amount of hydroxyproline. The counting efficiency for tritium was 10 percent. When enzyme was omitted, no more than 30 count/min of tritiated water was formed. Each value is the average of at least four determinations.

Organ	Protein extracted (mg/g)	Hydroxylase activity (cpm mg^{-1} hr^{-1} ±S.E.)	Total enzyme (cpm hr^{-1} g^{-1})
Heart	49	890 ± 130	43,710
Kidney	100	245 ± 22	24,500
Liver	140	235 ± 17	32,900
Lung	92	1145 ± 98	105,340
Muscle	40	250 ± 23	10,000
Skin	17	350 ± 35	5,950
Spleen	110	210 ± 12	23,100

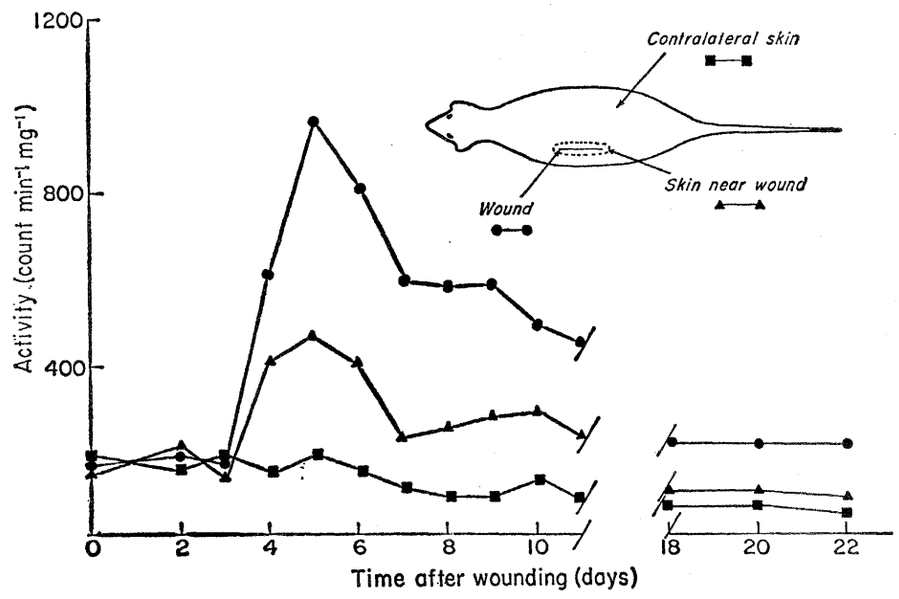

Fig. 2. Variations in the activity of collagen proline hydroxylase during healing of wounds in the rat (in counts per minute per milligram of protein). Each point is the average of at least four determinations. The total number of animals wounded was 80. The standard error at each point is approximately ± 15 percent of the mean.

produced in three separate groups of male rats. In a fourth experiment female rats were used. An identical time course was observed, but the maximum increase in enzyme was two- and threefold rather than fivefold. as in the males. Whether this difference between males and females is reproducible has not been determined.

The time course of events in the healing of wounds has been reviewed (*8*). During the first, or productive, phase (*8*) of healing, which lasts 4 to 5 days, there is a rapid increase in the number of fibroblasts as a result of cell migration and division but no increase in collagen, as measured by saline extractable hydroxyproline (*9*). [We would like to emphasize that measurements of hydroxyproline do not detect possible accumulations of nonhydroxylated protein or protocollagen (*1*).] An increase in extractable hydroxyproline first appears on days 4 to 5, which is a day or two later than the increase in peptidyl proline hydroxylase, and signals the onset of the second phase of wound healing (*8*). From this time until day 15 there is an increase of collagen in the wound and a parallel increase in tensile strength. Since there was no detectable increase in proline hydroxylase on day 3 (Fig. 2), although there are many fibroblasts at this time, it is probable that in the dividing or migrating fibroblast the level of hydroxylase is low. Whether dividing or migrating fibroblasts can synthesize a form of nonhydroxylated collagen and whether such material can be hydroxylated extracellularly have not been determined. The major enzymatic change that distinguishes the productive phase of wound healing from the collagen phase may be the induction in fibroblasts of collagen proline hydroxylase. This change may be associated with the transition of dividing, migrating cells to stationary, secretory cells.

The healing of a wound represents a relatively acute process. Chronic stimulation of the proliferation of fibroblasts and synthesis of collagen to form a granuloma can be induced with carrageenan (*6*). Rats were injected subcutaneously lateral to the spine with 0.5 ml of a 2-percent solution of carrageenan. A contralateral injection of 0.9 percent saline was given as a control. Animals were killed on different days and the specific activity of proline hydroxylase was determined in the injected areas (Fig. 3). Data from Benitz and Hall (*10*) are also plotted to show the time course of the accumulation of fibroblasts in the carrageenan granuloma. In the area injected with carrageenan, an increase in hydroxylase activity was observed by the 2nd day, which is 2 to 3 days before a comparable increase was observed in wounds. In the granuloma the increase was sustained for at least 28 days, whereas in wounds it had begun to decrease by the 6th day. Macroscopic evidence of the formation of granulomas could be seen on the 5th day.

Under different experimental conditions and at different stages of growth the specific activity and amount of collagen proline hydroxylase in the same tissue may vary over a severalfold range. Studies on the factors that control the levels of hydroxylase in tissues may eventually provide insight into the mechanisms that regulate the rate and amount of collagen biosynthesis by the fibroblast.

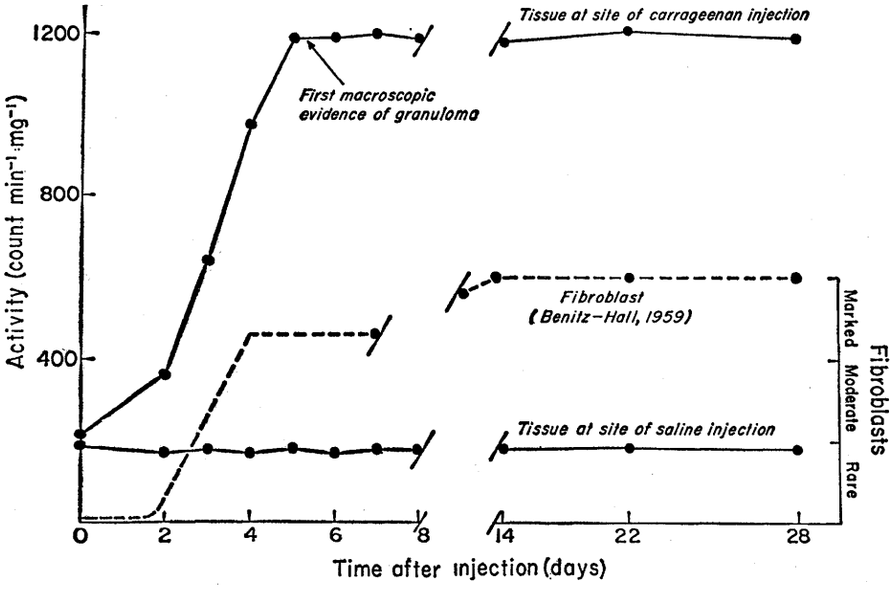

Fig. 3. Variatons in the activity of collagen proline hydroxylase during carrageenan granuloma formation in the rat (in counts per minute per milligram of protein). Each point is the average of at least four determinations. The total number of animals injected was 60. For days 0 to 5 the standard error at each point is approximately ± 30 percent of the mean; after day 5 the S.E. is approximately ± 10 percent of the mean. Curve ●-----● indicates the number of fibroblasts in the granuloma and is taken from the work of Benitz and Hall (*10*).

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References and Notes

1. The enzyme we have called collagen or peptidyl proline hydroxylase catalyzes the hydroxylation of certain prolyl residues in peptide linkage in hydroxyproline-deficient, collagenase-degradable protein to form collagen hydroxyproline (*2*). This protein accumulates under limiting conditions of hydroxylation and has a primary structure identical with collagen except for the replacement of hydroxyprolyl and hydroxylysyl by prolyl and lysyl residues. It has been suggested that the protein be called protocollagen and that the hydroxylating enzyme be called protocollagen hydroxylase (*3*). Protocollagen may not be an obligatory intermediate under normal conditions but can be converted to collagen by peptidyl proline hydroxylase.
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