*INDUCED CONNECTIVE TISSUE METABOLISM IN VIVO: REUTILIZATION OF PRE-EXISTING COLLAGEN**

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Light and electron microscopy of resorbing connective tissues have shown that the collagen fibers and fibrils disappear. It is usually presumed that resorbed collagen is degraded proteolytically to hydroxyproline-containing peptides and to free amino acids, including hydroxyproline. We wish to present evidence that collagen can be resorbed and then reutilized without such breakdown.

Urinary excretion of hydroxyproline in peptides has been regarded as an index of collagen catabolism *in vivo* because collagen is the only source of significant amounts of hydroxyproline.[1, 2] It has been suggested that a relatively constant fraction (20%) of the hydroxyproline from degraded collagen is excreted as peptides in urine.[2, 3] However, there is considerable evidence that under some conditions collagen is resorbed and the process is accompanied by minimal increases of excretion of peptide hydroxyproline. It has been observed that with increased bone resorption occurring in clinical[4] and experimental[5] hyperparathyroidism, there was increased urinary excretion of calcium unaccompanied by an increased excretion of peptide hydroxyproline. Likewise, in studies on the postpartum involution of the rat[6] and human uterus,[7] where massive resorption of collagen occurs within 1–8 days after parturition, a significant increase in urinary excretion of peptide hydroxyproline was not observed. These observations caused us to postulate that fibrous collagen might be depolymerized to a soluble subunit which may be reutilized without breakdown to free amino acids or low-molecular-weight peptides.[5, 7]

If collagen can be reutilized, it should be possible to demonstrate the contribution of pre-existing connective tissues to newly formed connective tissues by labeling the pre-existing collagen with radioactive proline *before* induction of the synthesis of new tissue. New connective tissues can be induced in animals by the subcutaneous injection of carrageenan,[8] or by subcutaneous implantation of polyvinyl sponges.[9] These techniques permit separation of newly formed collagen from pre-existing collagen and thus allow study of the new tissue without contamination with "old" tissue.

This approach necessitates a consideration of the possible pathways of reutilization[10] that may occur after labeling an intact animal with radioactive proline. Radioactivity in new tissues could arise from *de novo* synthesis using amino acid or peptide residues derived from either collagen or noncollagenous labeled protein. Alternately, reutilization could occur by the depolymerization (disaggregation) of collagen fibrils to soluble molecular units or subunits and subsequent extracellular repolymerization to fibrous collagen.

Since the hydroxylation of proline to hydroxyproline during collagen synthesis can be inhibited by vitamin C deficiency *in vivo*[8, 11] and *in vitro*,[12, 13] the possibility of a reaggregation pathway can be tested in prelabeled guinea pigs. If reutilization occurred by reaggregation of pre-existing labeled collagen subunits, the collagen

formed during vitamin C deficiency would have a higher specific activity than that of the normal animal, since the latter would form collagen not only by reaggregation using labeled subunits, but also by *de novo* synthesis from amino acids which would have a low specific activity.[10] A higher specific activity in vitamin C deficiency would indicate that collagen was the source of the reutilization rather than non-collagenous proteins, since hydroxyproline is not found in significant amounts in other proteins,[1] nor can hydroxyproline be utilized in *de novo* collagen synthesis.[14] If reutilization occurred by *de novo* synthesis from amino acids or peptides, the specific activity of collagen formed during vitamin C deficiency would be expected to be similar to that in the normal animals since synthesis in both cases would be from a similar precursor pool.

This communication presents evidence which indicates that there is significant reutilization of pre-existing label in the formation of "new" connective tissue, and suggests that this occurs by reutilization of the pre-existing collagen without breakdown to amino acids or low-molecular-weight peptides.

Materials and Methods.—Young female guinea pigs weighing 170–210 gm, young female Fischer rats weighing 56–74 gm, and young Sprague-Dawley rats weighing 84–94 gm were used. The animals were placed on Purina guinea pig and rat chow *ad libitum*.

Labeling procedure: Uniformly labeled L-proline-C^{14} was obtained from Schwarz BioResearch (175 mc/mM), and L-proline-3,4-H^3 from New England Nuclear Corp. (5 c/mM). During the active growing phase, each animal was given an intraperitoneal injection of radioactive proline (0.25 μc/gm), twice per week for 4 weeks. The guinea pigs received a total of 350–600 μc of H^3-proline, and the rats a total of 200–350 μc of C^{14}-proline per animal.

Induction of fibrous tissue in normal guinea pigs and rats: Three weeks after the last injection of H^3-proline, fibrous tissue was induced in guinea pigs by the subcutaneous administration of 5 ml of sterile 1% carrageenan in 0.9% NaCl in the abdominal region.[8] The guinea pigs were sacrificed 9–13 days after granuloma induction. The granuloma containing the "new" fibrous tissue was separated from overlying dermis and the abdominal wall by blunt dissection, adhering subcutaneous tissue was carefully removed, and contamination was minimized by leaving behind granuloma where it was contiguous with skin and abdominal musculature.

Histologic studies[15] confirmed that only minute remnants of muscle remained on the granuloma surface, where contamination with pre-existing tissue would be expected to be maximal, while the central areas of granuloma were free from skin or muscle.

Two and one-half weeks after the last injection of C^{14}-proline, three sterile polyvinyl sponges (Ivalon, Clay-Adams), measuring 1.5 × 1.5 × 0.7 cm (210–230 mg), were implanted subcutaneously under the dorsal skin of rats, using sodium pentobarbital anesthesia and aseptic technique. Before implantation, the sponges were soaked in sterile isotonic saline.[9] The rats were sacrificed 21 days after sponge implantation. Polyvinyl sponges were removed from their subcutaneous pockets with a minimum of dissection. They were covered on all sides with a fibrous capsule which could be easily stripped off. One sponge from each rat was used for characterization of the newly formed collagen, the second sponge for a comparison of sponge interior and sponge capsule, and the third sponge was fixed in neutral formalin for histology and autoradiography.

Induction of fibrous tissue in scorbutic guinea pigs: Female guinea pigs were labeled with H^3-proline as above. After 39 days, five guinea pigs were placed on a vitamin C-deficient diet (Reid-Briggs diet, General Biochemicals, Chagrin Falls, Ohio), and five animals were placed on a normal diet as controls. After 49 days, polyvinyl sponges were implanted subcutaneously on the dorsum of all animals; and 60 days after labeling, 5 ml of 1% carrageenan was administered subcutaneously in the abdomen of two normal and two scorbutic guinea pigs. Animals were sacrificed 71 days after labeling.

Purification of induced granulation tissues: Polyvinyl sponges and weighed portions of granulomas were minced and then gelatinized by autoclaving in 10 ml of water for 1 hr at 120°. After centrifugation at 25,000 × *g* for 15 min at room temperature, the solution containing the gelatin was dialyzed (Visking 20/32 dialysis tubing) against cold running tap water overnight. Tri-

chloroacetic acid (TCA) was added to the nondialyzable gelatin solutions to a final concentration of 5% and they were heated in a water bath at 90° for 30 min.[16] The flocculent precipitate was removed by centrifugation as before and was discarded.

Analyses: Aliquots of gelatin, weighed portions of carrageenan granulomas, and samples of skin and bone were hydrolyzed in 6 *N* HCl for 3 hr at 120°. Proline and hydroxyproline were separated by ion-exchange chromatography.[4] Recovery of proline and hydroxyproline is 94–98% by this method. The hydroxyproline and proline fractions are free of other amino acids although a small amount of glutamic acid is found in the late end of the proline eluate (see Fig. 1). Hydroxyproline analyses were done by a modification[17] of the Neuman and Logan procedure,[18] and proline analyses by the method of Summer and Roszel.[19]

Radioactive procedures: Radioactivity was determined on aliquots of gelatins, hydrolysates, and chromatographic eluates in a liquid scintillation spectrometer, using the scintillation liquid described by Wheelock.[21] C^{14} counting efficiencies (70%) were monitored by the channels ratio method,[22] and H^3 counting efficiencies (24%) were determined by internal standards. Observed counts per minute were converted to disintegrations per minute. Eluates containing proline or hydroxyproline were collected in 5-ml fractions, and specific activities in the fractions agreed to at least ± 5%. Constancy of specific activity in fractions of the eluates showed that there was no radioactive contamination of hydroxyproline and proline (Fig. 1).

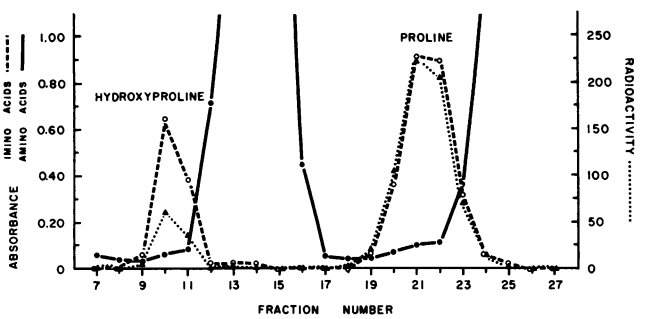

FIG. 1.—Chromatographic separation of H^3-hydroxyproline and H^3-proline from other amino acids of granuloma hydrolysate. One-ml aliquots of each 5-ml fraction were analyzed colorimetrically for hydroxyproline[17, 18] at 540 mμ, proline[19] at 515 mμ, and other amino acids by the ninhydrin reaction[20] at 570 mμ, and were analyzed for total radioactivity (cpm). Specific radioactivity was determined from fractions 10 and 11 for hydroxyproline and fractions 21 and 22 for proline. The slight increase of ninhydrin[20] absorbance in the hydroxyproline and proline fractions is due to their weak absorbance at 570 mμ.

Calculations: Total collagen was calculated from the following formula: mg collagen = mg hydroxyproline × 7.46.[1] For calculations of ratios of the molar specific activity of proline to hydroxyproline in H^3-labeled tissue, hydroxyproline specific activity was multiplied by 1.33 to correct for the loss of one atom of H^3 from proline[23] due to hydroxylation. Total radioactivity was obtained by determining the specific activity in peak fractions of the eluates and calculating total activity from hydroxyproline and proline analysis.

Results.—Characterization of induced connective tissues: Total collagen was 30–47 mg per polyvinyl sponge and 22–150 mg per carrageenan granuloma (Table 1). After dialysis and hot TCA treatment of the gelatinized granulation tissues, the average molar ratio of proline to hydroxyproline was 1.31 for sponge collagen and 1.35 for granuloma collagen (Table 1), which is consistent with those reported for purified collagen (1.18 to 1.45).[24] The average ratio of the molar specific activity of proline to hydroxyproline after dialysis and hot TCA treatment of the gelatins was 0.91 for sponge collagen, and 1.28 for granuloma collagen (Table 1) which is

consistent with those reported for acutely labeled collagen (0.85–1.15).[25, 26] Proline and hydroxyproline accounted for most of the radioactivity present (an average of 77.1 per cent for sponge collagen, and 89.3 per cent for granuloma collagen). The per cent of total radioactivity that was dialyzable averaged 8.1 per cent for sponge collagen, and 9.8 per cent for granuloma collagen.[27] An average of 9.9 per cent of the total radioactivity of sponge collagen was precipitated by treatment with 5 per cent TCA (final concentration) at 90°, while an average of 2.9 per cent was precipitated from granuloma samples (Table 1). These data indicate that the tissues were not contaminated with significant free amino acid, peptide, or noncollagenous protein-bound radioactivity.

TABLE 1

CHARACTERIZATION OF INDUCED CONNECTIVE TISSUES

	Rat no.	Guinea pig no.	Total collagen (mg)	Proline/Hydroxyproline Ratios* Molar	Molar specific activity	Distribution of Total Radioactivity Per cent of total as pro & hypro	Per cent dialyzable	Per cent insoluble in 5% TCA	Per cent soluble in 5% TCA
Polyvinyl sponge	F1	—	29.8	1.28	0.90	77.1	12.9	5.8	81.2
	F2	—	37.3	1.33	0.80	69.0	5.3	14.7	78.1
	F3	—	40.3	1.36	0.88	79.8	6.1	14.3	79.4
	SD-4	—	38.0	1.33	0.89	70.0	4.0	8.6	87.3
	SD-5	—	43.3	1.29	0.81	79.8	6.2	10.0	82.9
	SD-6	—	47.0	1.29	1.18	87.2	13.8	5.9	80.1
Carrageenan granuloma	—	1	21.5†	—	—	80.2	—	—	—
	—	2	44.6	1.42	1.38	95.4	9.2	1.7	89.0
	—	3	74.5	1.32	1.21	93.1	9.6	7.0	83.5
	—	4	133.	1.39	1.09	88.7	10.9	0	92.4
	—	5	150.	1.28	1.44	89.0	35.7‡	4.7	59.5‡

* After purification of extracted gelatin.
† All guinea pigs gained weight during the period of granuloma formation except no. 1.
‡ Suspected leak in dialysis tubing.

Radioactivity in induced connective tissues: Tables 2 and 3 compare specific activity of hydroxyproline in sponge and granuloma connective tissues with hydroxyproline isolated from skin and bone. Hydroxyproline specific activity from sponge connective tissue (Table 2) was 30–54 per cent (average = 41%) of that observed in skin from a site distal to the sponge, and was 12–20 per cent (average = 15%) of that observed in the diaphysis of the femur. After dialysis and hot TCA treatment of the gelatin extracts these data were 36–67 per cent (average = 52%) and 12–26 per cent (average = 21%). These values were obtained from a sponge without removal of capsule. After removal of capsule from a separate sponge, hydroxyproline specific activity was 16–31 per cent (average = 21%) of that observed in distal skin. Since the origin of the capsular material is not certain, these figures may represent the specific activity of the connective tissue which is, in fact, newly formed. Specific activity of hydroxyproline isolated from the capsule was 2.5 to 4 times greater than that isolated from the interior of the sponge (Table 2), suggesting the possibility of contribution of subcutaneous tissues to capsule formation. Autoradiographs[15] demonstrated activity in the central and peripheral areas of the sponge and confirmed that the capsular tissue was more radioactive.

The specific activity of hydroxyproline isolated from granuloma samples (Table 3) after hydrolysis of an aliquot of tissue was 59–109 per cent (average = 85%) of that observed in distal skin, and 25–103 per cent (average = 71%) of that observed

TABLE 2

SPECIFIC C^{14} ACTIVITIES OF HYDROXYPROLINE ISOLATED FROM CONNECTIVE TISSUES IN CHRONICALLY LABELED RATS

	Specific Radioactivity (dpm/μg hydroxyproline)							
	Sponge No. 1		Sponge No. 2		Skin		Bone	
Animal no.	Unpurified	Purified	Purified		Over sponge	Distal site*	Femur epiphysis	Femur diaphysis
			Sponge	Capsule				
F1	6.0	6.4	3.1	8.1	11.6	14.9	22.6	30.6
F2	1.4	2.3	0.75	3.1	3.8	4.7	6.3	10.9
F3	3.1	4.3	1.5	5.6	5.5	7.9	13.0	21.2
SD-4	6.4	7.9	3.7	10.2	10.9	11.8	22.2	30.9
SD-5	5.3	7.3	2.0	6.8	10.0	11.8	17.4	31.7
SD-6	4.7	4.8	2.8	7.0	12.7	13.3	23.0	39.4

* Taken from the abdominal region.

TABLE 3

SPECIFIC H^3 ACTIVITIES OF HYDROXYPROLINE ISOLATED FROM CONNECTIVE TISSUES IN CHRONICALLY LABELED GUINEA PIGS

	Specific Radioactivity (dpm/μg hydroxyproline)					
	Granuloma		Skin		Bone	
Animal no.	Unpurified*	Purified*	Over granuloma	Distal site†	Femur epiphysis	Femur diaphysis
1‡	17.3	—	14.8	15.6	13.4	17.9
2	9.0	7.1	—	7.8	7.2	11.7
3	3.4	9.2	10.1	10.8	9.5	13.4
4	6.6	9.1	9.1	11.1	9.6	13.0
5	15.3	11.3	13.0	14.0	10.9	14.8

* Different portions of the same granuloma.
† Taken from the dorsum.
‡ All guinea pigs gained weight during the period of granuloma formation except no. 1.

TABLE 4

SPECIFIC H^3 ACTIVITIES OF HYDROXYPROLINE ISOLATED FROM CONNECTIVE TISSUES IN CHRONICALLY LABELED NORMAL AND SCORBUTIC GUINEA PIGS

		Collagen		Specific Radioactivity (dpm/μg hydroxyproline)			
Guinea pig no.	Diet	Sponge (mg)	Granuloma (mg)	Sponge	Granuloma	Skin	Bone
1	Normal	13.0	—	1.9	—	6.1	5.3
2	"	9.4	—	2.1	—	7.4	7.4
3	"	12.7	168.	1.4	3.3	7.2	5.6
4	"	3.5	—	1.6	—	7.1	7.2
5	"	4.1	107.	1.7	3.9	7.9	7.3
6	Vitamin C-deficient	0.74	—	8.9	—	10.8	8.2
7	"	0.52	—	6.2	—	8.3	8.1
8	"	0.54	—	6.6	—	9.9	9.3
9	"	0.41	10.1	7.5	24.0	8.6	7.1
10	"	0.87	18.7	6.5	20.4	10.7	10.6

in the femur diaphysis. Gelatinization, dialysis, and hot TCA treatment of another portion of granuloma gave data of 81–124 per cent (average = 97%) and 69–83 per cent (average = 78%), respectively. Autoradiographs[15] demonstrated that central and peripheral areas were labeled.

Radioactivity in induced connective tissues in scorbutic guinea pigs: In scorbutic animals (Table 4), the total amount of collagen in sponges and granulomas averaged 7.4 and 10 per cent of their nonscorbutic controls. Specific activity of hydroxyproline from sponge connective tissue of normal guinea pigs was 22–31 per cent (average = 25%) of that observed in skin from a distal site and in sponges of scorbutic animals was 61–87 per cent (average = 74%). Specific activity of hydroxyproline from granulomas of normal guinea pigs was 46 and 50 per cent of that observed in skin from a distal site, and in granulomas in scorbutic animals it was 190 and 280 per cent.

Discussion.—The animals in this study were labeled during the period of active growth for a period of 4 weeks (see labeling procedure) to obtain maximum efficiency of incorporation[28] and a relatively even distribution of radioactivity in collagens of various "ages." Since the half life of parenterally injected C^{14}-proline has been shown to be less than 24 hr,[29] a 2.5–3-week interval was allowed between the last injection of radioactive proline and induction of granulation tissue so that radioactive proline from the injections would not be present when the induced tissues were developing.

The techniques[8, 9] used for inducing growth of connective tissue minimized contamination by pre-existing radioactive tissues. This was confirmed histologically[15] by the absence of recognizable skin or muscle in the tissues. If the radioactivity observed in hydroxyproline and proline were from contamination with pre-existing tissues, autoradiographs should give a distinctive pattern showing new tissue without significant labeling interspersed with areas of highly labeled tissue. However, autoradiographs[15] demonstrated an even pattern of labeling both in sponge and granuloma tissue, which is inconsistent with contamination. The validity of the autoradiographic observations is supported by the findings that most of the radioactivity was found in the proline and hydroxyproline of collagen. The molar specific activity and molar ratios of proline to hydroxyproline are characteristic for normal collagen labeled by the acute injection of radioactive proline.[24–26] This indicated that the newly formed collagen had a normal proline and hydroxyproline composition and was labeled in a physiologic manner.

The 2.5–3-week period between the last injection of radioactive proline and induction of the granulation tissues should preclude the availability of exogenous (from injections) radioactive proline for labeling the new tissues. Therefore, it was concluded that the newly formed tissues were labeled by reutilization of endogenous radioactivity previously incorporated into body protein. This phenomenon could occur by a synthetic[30] or reaggregation pathway. The findings of a four- to sixfold greater specific radioactivity in hydroxyproline isolated from sponges and granulomas of scorbutic guinea pigs as compared to sponges and granulomas of nonscorbutic guinea pigs (Table 4) support the suggestion[10] that the formation of newly formed radioactive collagen does not occur by *de novo* synthesis from amino acids and support the view that the source of radioactivity is pre-existing collagen subunits.[10] Noncollagenous proteins do not contain significant amounts of hydroxyproline,[1] and therefore could not label collagen hydroxyproline in granulation tissue by transfer.

A pathway involving reaggregation is consistent with present concepts of fibrous collagen formation. Collagen is synthesized intracellularly[31] as soluble units which are then secreted and polymerized extracellularly to form collagen fibrils.[32, 33] The reutilization phenomenon indicated by the present work could occur by the depolymerization (or disaggregation[34]) of fibrous collagen (aggregates of macromolecules) to soluble molecular units or subunits and the subsequent extracellular polymerization of fibrous collagen[3, 7] without initial breakdown to free amino acids or low-molecular-weight peptides.

Classical isotopic turnover studies measure a *net* change in isotope content, and have demonstrated that mature fibrous collagen normally loses incorporated isotope slowly,[35–37] leading to the conclusion that mature collagen is relatively inert meta-

bolically.[35-37] Since reutilization may result in no net change in radioactivity, turnover of fibrous collagen via reutilization of subunits would be consistent with the slow loss of isotope observed, but would indicate that the metabolic inertness of collagen is only apparent.

Summary.—Hydroxyproline isolated from newly formed connective tissues of normal animals previously labeled with radioactive proline contained significant specific radioactivity when compared to other connective tissues. The formation of newly formed collagen clearly involved reutilization of previously incorporated radioactivity.

Hydroxyproline isolated from newly formed connective tissues of animals made scorbutic after previous labeling had a higher specific radioactivity than that of collagen from the nonscorbutic controls. Since hydroxylation of proline has been shown by others to be inhibited by vitamin C deficiency, this reutilization apparently did not involve *de novo* synthesis. It is postulated that pre-existing labeled collagen was depolymerized to molecular units or subunits and that these units were then reaggregated to contribute to newly formed radioactive connective tissues.

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