REUTILIZATION OF MATURE COLLAGEN IN VIVO[+]

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Received October 8, 1965

Studies in this laboratory have suggested that mature collagen might be reutilized (Klein et al., 1962, 1964; Klein, 1964). The induction of granulation tissue in animals previously labeled with radioactive proline would determine whether mature fibrous collagen is reutilized in the formation of "new" connective tissue, because only the pre-existing collagen would be radioactive.

MATERIALS AND METHODS

Animals. Young female guinea pigs weighing 220-250 g, young female Fischer rats weighing 56-74 g, and young female Sprague-Dawley rats weighing 84-94 g 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 microcurie/g), twice per week for 4 weeks. The guinea pigs received a total of 350-600 microcuries

[+] This investigation was supported in part by Public Health Service Research Grants HD-00669 and FR-5410 from the Institutes of Child Health and Human Development and General Medical Sciences, and Rainbow Hospital Research Fund.

of H^3-proline, and the rats a total of 200-350 microcuries of C^{14}-proline per animal.

Induction of fibrous tissue. Two techniques were used which permit accurate distinction between newly formed and adjacent old tissues: (1) subcutaneous carrageenan injection (Robertson and Schwartz, 1953) and (2) polyvinyl sponge implants (Edwards et al., 1957).

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. 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, and any adhering subcutaneous tissue was carefully removed.

Two and one-half weeks after the last injection of C^{14}-proline, sterile polyvinyl sponges (Ivalon, Clay Adams), measuring 1.5 x 1.5 x 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. The rats were sacrificed 21 days after sponge implantation, and the sponges were removed in the same manner as the carrageenan granulomas.

Analyses. Collagen in polyvinyl sponges was gelatinized by autoclaving the minced sponge in water for 1 hour at 120°C. Aliquots of this gelatin and weighed portions of carrageenan granulomas were hydrolyzed in 6N HCl for 3 hours at 120°C. Samples of "old collagen" (skin and bone) were hydrolyzed in the same manner. Proline (pro) and hydroxyproline (hypro) were

separated by ion-exchange chromatography (Klein et al., 1962). Recovery of pro and hypro is 94-98% by this method. The hypro peak is free of other amino acids and a small amount of glutamic acid is found in the tail of the pro peak. Hypro analyses were done by a modification (Klein, 1958) of the Neuman and Logan procedure (1950), and pro analyses by the method of Summer and Roszel (1965).

Radioactive procedures. Radioactivity was determined on aliquots of the chromatographic eluates that were used for chemical analysis, and hydrolyzates in a liquid scintillation spectrometer. C^{14} counting efficiencies were monitored by the channels ratio method (Baillie, 1960) and H^3 counting efficiencies were determined by internal standards. Observed counts per minute were converted to disintegrations per minute. Determination of specific activity in each fraction of the eluates showed that there was no radioactive contamination of hypro, and a minimal amount in the tail of the pro eluate. Total activity of pro was obtained by determining specific activity in early fractions of the eluate which were free from contamination, and calculating total activity from pro analyses.

RESULTS AND DISCUSSION

The total collagen* on the basis of hypro content was 22-150 mg per carrageenan granuloma and 30-47 mg per polyvinyl sponge.

The specific radioactivity of hypro in the granuloma collagen was 32-115% (average = 86%) of that observed in skin from a site distal to the granuloma, and was 25-99% (average = 70%) of that observed in the diaphysis of the femur (Table I). The average molar specific activity ratio of pro/hypro was 1.27.

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* mg collagen = mg hydroxyproline x 7.46 .

The specific radioactivity of the hypro in the sponge connective tissue was 30-54% (average = 41%) of that observed in the skin from a site distal to the sponge, and was 12-20% (average = 16%) of that observed in the diaphysis of the femur (Table II). The average molar specific activity ratio of pro/hypro was 1.11.

The presence of significant amounts of radioactivity in hypro of induced connective tissues under the experimental conditions described here could be explained by a number of possibilities: (1) utilization of exogenous (from injections) or endogenous (from the catabolism of collagen) radioactive proline for de novo collagen synthesis, (2) reutilization of free radioactive hydroxyproline (from catabolism of collagen), (3) reutilization of hypro-containing peptides, and (4) reutilization of mature collagen.

TABLE I*

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

Animal	Collagen per granuloma (mg)	SPECIFIC RADIOACTIVITY (dpm/microgm hydroxyproline)					
		Granuloma	Skin		Bone		
			Over granuloma	Distal site	Right femur epiphysis	Right femur diaphysis	Left femur diaphysis
1*	21.5	17.3	14.8	15.6	13.4	17.9	17.4
2	44.6	9.0	—	7.8	7.2	11.7	11.5
3	74.5	3.4	10.1	10.8	9.5	13.4	13.8
4	133.	6.6	9.1	11.1	9.6	13.0	12.3
5	150.	15.3	13.0	14.0	10.9	14.8	16.0

* All guinea pigs gained weight during experimental period except #1

TABLE II+

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

Animal	Collagen per sponge (mg)	SPECIFIC RADIOACTIVITY (dpm/microgm hydroxyproline)				
		Sponge	Skin		Bone	
			Over sponge	Distal site	Femur epiphysis	Femur diaphysis
F1	29.8	6.0	11.6	14.9	22.6	30.6
F2	37.3	1.4	3.8	4.7	6.3	10.9
F3	40.3	3.1	5.5	7.9	13.0	21.2
S-D4	38.0	6.4	10.9	11.8	22.2	30.9
S-D5	43.3	5.3	10.0	11.8	17.4	31.7
S-D6	47.0	4.7	12.7	13.3	23.0	39.4

+ Before chromatographing each granuloma or sponge sample, the chromatographic columns were assayed for radioactivity and none was observed. All hypro samples were fractionated on the columns, and specific radioactivities in the fractions agreed to at least ± 5%. Counts per minute of H^3-hypro from granuloma samples were at least 3 and usually 5-10 times background, and counts per minute of C^{14}-hypro from sponge connective tissue samples were at least 4 and usually 8-18 times background.

The half life of parenterally injected C^{14}-proline *in vivo* has been shown to be less than 24 hours (Lindstedt and Prockop, 1961), indicating that the 2.5-3 week period between the last injection of radioactive proline and induction of the granulation tissues precluded the availability of exogenous radioactive proline for labeling the "new" tissue. The data of Lindstedt and Prockop (1961) indicate that there is no significant relabeling of collagen hypro from the breakdown of proteins containing C^{14}-proline.

Stetten (1949) found that orally administered N^{15}-hypro was not significantly incorporated into collagen, nor is parenterally administered C^{14}-hypro (Wolf et al., 1956). There is little evidence for the exchange of amino acids with intact proteins (Hoagland, 1960), or for the reutilization of peptides for protein synthesis (Borsook, 1956; Fruton, 1963).

In order to determine whether free or peptide-bound radioactivity would be reutilized under the experimental conditions employed, chronically labeled female rats were made pregnant in place of inducing granulation tissue. Specific radioactivity of total fetal hypro at 19 days of pregnancy was 3.5% and 1.6% of that observed in maternal skin and bone, respectively, indicating that insignificant amounts of radioactivity were reutilized for fetal de novo collagen synthesis*. This suggested that induced granulation tissue would not contain significant amounts of radioactive hypro if de novo synthesis were the only source of collagen.

It was concluded that pre-existing collagen was reutilized in the formation of the "new" fibrous tissue. The high specific radioactivity of the carrageenan granuloma hypro in relation to other tissues suggests that reutilization was the major pathway in the formation of the granuloma. The data from polyvinyl sponge implants suggest that de novo synthesized collagen made the greater contribution to this tissue.

The reutilization of collagen implies a cyclic turnover which would be consistent with the apparent metabolic inertness of mature fibrous collagen.

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* Klein, L., and Teree, T. M., unpublished observations.

ACKNOWLEDGMENT

The excellent technical assistance of Mrs. Corinne Pulphus is gratefully acknowledged.

REFERENCES

Baillie, L. A., Intern. J. Appl. Rad. Isotopes 8, 1 (1960).
Borsook, H., J. Cell. Comp. Physiol. 47, Suppl. 1, 47 (1956).
Edwards, L. C., Pernokas, L. N., and Dunphy, J. E., Surg. Gyn. Obs. 105, 303 (1957).
Fruton, J. S., in The Proteins, 2nd edition, H. Neurath (Ed.), Academic Press, New York, Vol. 1, 1963, p. 254.
Hoagland, M. B., in The Nucleic Acids, E. Chargaff and J. N. Davidson (Eds.), Academic Press, New York, Vol. 3, 1960, p. 351.
Klein, L., Ph. D. Thesis, Boston University, 1958.
Klein, L., Metabolism 13, 386 (1964).
Klein, L., Albertsen, K., and Curtiss, P. H., Jr., Metabolism 11, 1023 (1962).
Klein, L., and Curtiss, P. H., Jr., in Dynamic Studies of Metabolic Bone Disease, O. H. Pearson and G. F. Joplin (Eds.), Blackwell, Oxford, 1964, p. 201.
Lindstedt, S., and Prockop, D. J., J. Biol. Chem. 236, 1399 (1961).
Neuman, R. E. and Logan, M. A., J. Biol. Chem. 184, 299 (1950).
Robertson, W. van B., and Schwartz, B., J. Biol. Chem. 201, 689 (1953).
Stetten, M. R., J. Biol. Chem., 181, 31 (1949).
Summer, G. K., and Roszel, N. O., Clin. Chem. 11, 455 (1965).
Wolf, G., Heck, W. W., and Leak, J. C., J. Biol. Chem. 223, 95 (1956).