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Incorporation and Conversion of Lysine-2-C^{14} in Rat Biopsy-Connective Tissue. (24095)

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The amino acids, hydroxyproline and hydroxylysine, are found chiefly in collagen and are responsible in part for stability of the fiber(1,2). Hydroxyproline cannot be incorporated directly into the peptide chain of collagen, but a portion of the proline becomes hydroxylated following its incorporation(3). Presumably lysine and hydroxylysine are involved in collagen formation in a similar manner because only labeled lysine fed to an animal is incorporated into collagen and a portion appears as hydroxylysine(4).

The present report deals with incorporation, distribution and rate of turnover of C^{14}-labeled lysine in rat connective tissue obtained by sponge implantation technic(5). Conversion of labeled lysine to hydroxylysine in connective tissue of different ages and from both sexes is also presented.

Methods. Sprague-Dawley rats of approximately 6 months of age and weighing 200 to 250 g were used. All animals included were given a single intraperitoneal injection of 1 ml of neutralized dl-lysine-2-C^{14} monohydrochloride (9.45 mg or 0.024 mc) in physiological saline. Three polyvinyl sponges (Ivalon†) were implanted subcutaneously along dorsum of rat. Previous histological work indicates growth of normal appearing connective tissue into the interstices of the sponge(5). The fact that rate of collagen formation in sponge biopsy(6) was similar to that found by Abercrombie *et al.* in wound healing in the rat(7) suggests comparable metabolic rates for the 2 connective tissues. Incorporation of labeled lysine and formation of tagged hydroxylysine were studied in connective tissue of 2 male and 2 female rats, 21 days after sponge implantation and in one male and one female rat 300 days after implantation. The tissues were removed 16 hours following intraperitoneal injection. Sponge-tissues were weighed, homogenized with 10 ml of physiological saline in VirTis "45" Macro Homogenizer‡ for 5 minutes with rheostat reading at 50, and centrifuged at approximately 15,000 x g at 5°C for 1 hour to obtain the saline-soluble protein. The residue was washed with distilled water and centrifuged at approximately 15,000 x g at 5°C for 30 minutes, and the wash discarded. The resultant residue was shaken with 5 ml of 0.5 M acetic acid at 5°C for three 24-hour periods in 12 ml plastic tube with rubber stopper. After each 24-hour period, the plastic tubes were centrifuged at 90,000 x g in a Spinco Model E ultracentrifuge for 1 hour and the acetic acid-soluble protein decanted. The 3 acetic acid extracts were combined. Protein of the saline-soluble fraction was precipitated with 4 volumes of 10% trichloroacetic acid. The trichloroacetic acid precipitate of the saline, the acetic acid-soluble protein and the residue were hydrolyzed with 6 N HCl in sealed pyrex tubes at 110°C for 16 hours. Nitrogen was determined by Conway microdiffusion technic(8) and converted to a protein value by factor 6.25. An aliquot of the hydrolysate was evaporated to dryness on steam bath to remove excess HCl, neutralized and again evaporated to dryness. The neutralized samples were dissolved in citrate buffer, pH 5.0, to concentration of 15-20 mg

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of protein/ml, and 1 ml was applied to a 15 cm column of Dowex 50-x 8 (200-400 mesh). The method of Moore and Stein(9) for basic amino acid chromatography was used. The column was eluted first with 30 ml of citrate buffer, pH 5.0, and this eluate was discarded. A phosphate buffer, pH 6.8, was then used, and the eluate was collected in 2 ml fractions. An aliquot of each fraction was placed in aluminum planchet and evaporated to dryness for radioactivity measurement, and an aliquot was used for the ninhydrin reaction(10). The effects of age of tissue and of sex of donor rat upon incorporation of lysine and synthesis of hydroxylysine were studied in 17-, 50- and 300-day old tissues from female rat and in 17-, 90- and 300-day old tissues of the male, which were removed 16 hours after lysine injection. Protein of connective tissue biopsies was partitioned into saline-soluble and -insoluble fractions as previously described. The saline-insoluble residue was then divided into collagen and non-collagenous protein by the method of Fitch, who reported 95 to 100% extraction of collagen with hot (90°C) trichloroacetic acid extraction(11). Similar extraction results were found from the sponge-connective tissue. All fractions were hydrolyzed with 6 N HCl in sealed pyrex tubes for 16 hours at 110°C, and neutralized aliquots of the hydrolysates were taken for column chromatography and analysis as described. Turnover rates of lysine- and hydroxylysine-containing proteins of biopsy-connective tissue were followed in a female rat and in 2 male rats. The animals, which, at age of 4 to 6 months, had been previously implanted with 2 or 3 sponges, were given intraperitoneal injection of labeled lysine, and connective tissue biopsies were removed at different intervals of time following injection. The connective tissue-sponges in the female, which were 50 days of age at time of injection, were removed at 8 hours, 17 and 31 days following lysine injection. Tissues in male rats were 90 days old at time of injection and were removed after 8 hours and 11 days. The tissues were fractionated into saline-soluble protein, collagen and non-collagenous protein. Nitrogen and radioactivity were determined on neutralized acid hydrolysates of tissue fractions, and the specific activity (total cpm/mg of protein) was calculated.

TABLE I. Lysine and Hydroxylysine in Rat Biopsy-Connective Tissue.

	Male		Female	
	21-day*	300-day	21-day*	300-day
Amino acid conc. (% of nitrogen)				
Hydroxylysine				
Saline-soluble protein	0	0	0	0
Acetic acid-soluble protein	.36	.35	0	.94
Residue	.60	.85	.62	.60
Lysine				
Saline-soluble protein	7.43	8.90	9.82	8.41
Acetic acid-soluble protein	5.53	7.54	6.79	5.10
Residue	5.16	4.76	4.72	4.99
Specific activity (cpm/mg of amino acid)				
Hydroxylysine				
Acetic acid-soluble protein	940	264		322
Residue	541	246	990	446
Lysine				
Saline-soluble protein	953	878	849	897
Acetic acid-soluble protein	1180	629	892	850
Residue	1073	282	1182	1278

* Mean of 2 values.

Tissues were removed 16 hr after inj. of dl-lysine-2-C^{14} monohydrochloride (9.45 mg or 0.024 mc).

Results. Concentrations and specific activities of lysine and hydroxylysine in saline-soluble protein, acetic acid-soluble protein and the residue of biopsy-connective tissue 16 hours after injection of lysine-2-C^{14} are shown in Table I. Hydroxylysine was not found in the saline-soluble protein of biopsy-connective tissue. In male biopsy-connective tissue, hydroxylysine concentration was lower in the acetic acid-soluble protein than in the residue and was lower in the residue of 21-day than in that of 300-day old tissue. Hydroxylysine was apparently not present in the acetic acid-soluble protein of the tissue from the female at 21 days but was present in this fraction in the tissue of 300 days of age. Hydroxylysine concentrations in the acetic acid-insoluble residue of female biopsy-connective tissue of 21 and 300 days of age were not different. Ly-

TABLE II. Incorporation and Conversion of Lysine-2-C^{14} in Biopsy-Connective Tissue.

	Lysine		Hydroxylysine	
	%*	S.A.†	%	S.A.
17-day tissue				
Collagen				
♂	3.22	858	.67	939
♀	3.09	784	1.02	804
Non-collagen protein				
♂	9.73	739	.0	0
♀	7.84	1074	.0	0
50-day female tissue				
Collagen	3.67	485	.94	393
Non-collagen protein	10.12	1211	.0	0
90-day male tissue				
Collagen	3.47	332	.81	303
Non-collagen protein	11.46	1007	.0	0
300-day tissue				
Collagen				
♂				246‡
♀				446‡

* Grams of amino acid/100 g of protein.
† S.A. = Specific activity (cpm/mg amino acid).
‡ Data from acetic acid fractionation. Since hydroxylysine exists only in collagen fraction, these data, calculated on basis of hydroxylysine concentration, are used for comparison.

sine concentration was greatest in the saline-soluble fraction and lowest in the residue of the 21-day and 300-day old tissues of both sexes.

Specific activities of hydroxylysine (cpm/mg of amino acid) in the acetic acid-soluble protein and residue were lower in the 300-day old tissue than in the 21-day old tissue in both sexes, with the exception of the acetic acid-soluble protein of female tissue in which there was no measurable amount for comparison at 21 days of tissue age. Specific activity of lysine did not decrease with tissue age in any fraction of female tissue, but in the male there was a reduction in activity of both fractions of saline-insoluble residue of tissue from 21 to 300 days of age.

The acetic acid-soluble and -insoluble fractions consist of collagen and a non-collagenous protein(6). Determination of specific activity of lysine and of hydroxylysine in these 2 proteins of tissue, which were separated by hot trichloroacetic acid extraction of the collagen from saline-insoluble residue of male and female connective tissue, indicated that hydroxylysine was found entirely in the collagen. The labeled lysine was found in the collagen, the non-collagenous protein and in saline-soluble protein. Of the total protein of tissue, 36.4% was in the collagen, 21.8% in the non-collagenous fraction and 41.8% in the saline-soluble fraction.

The effects of tissue age and of sex of donor rat upon incorporation of lysine-2-C^{14} and its conversion to labeled hydroxylysine in biopsy-connective tissue are shown in Table II. In the collagen, specific activity of both lysine and hydroxylysine decreased with increase in tissue age from 17 to 50 days in the female and from 17 to 90 days in the male. This decrease appeared to be more marked in the male. Specific activity of hydroxylysine of the 300-day old female tissue was twice that of similarly aged male tissue. There was no parallel reduction in specific activity of lysine with tissue age in the non-collagenous protein in tissue of either sex.

Concentrations and radioactivities of the basic amino acids of saline-soluble protein, acetic acid-soluble protein and residue are shown in Fig. 1. Only the basic amino acids contained radioactivity. Radioactivity in the saline-soluble protein was confined to lysine. The acetic acid-soluble protein and the residue contained tyrosine, phenylalanine, histidine, hydroxylysine and lysine.

The composite radioactivity decay curves for saline-soluble, collagen and non-collagenous fractions of tissues obtained from male and female rats are plotted as specific activity (total cpm/mg of protein) against time in days in Fig. 2. Radioactivity in non-collagenous and saline-soluble fractions rose promptly during the first 8 hours and decayed during the following 10 days to less than 40% of the 8-hour value. Rate of decay then slowed so that the activity at 30 days was approximately 10% of maximum. Maximum activity of the collagen was reached within 8 hours following injection of the isotope but rose to only 30% of maximum specific activity of the non-collagenous protein. Decay of activity in the collagen occurred more slowly than in the other 2 fractions, and at 30 days, 30% of the activity remained.

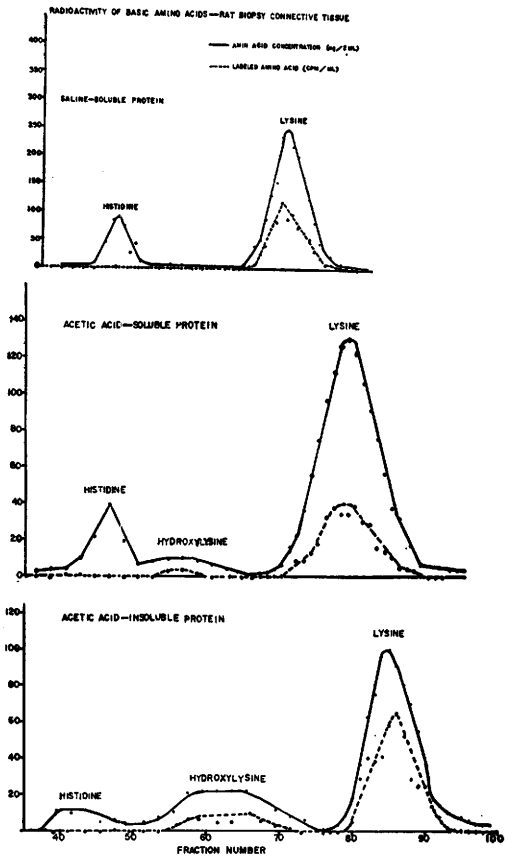

FIG. 1.

Discussion. Incorporation of lysine-2-C^{14} into sponge-biopsy connective tissue and conversion of a portion of tagged lysine to hydroxylysine has been demonstrated. Labeled lysine administered intraperitoneally becomes incorporated within 8 hours into protein of ground substance (saline-soluble fraction), into non-collagenous protein and into collagen. In recent experiments not included in this report labeled lysine and hydroxylysine were found in the collagen within 3 hours following intraperitoneal injection.

Rapid incorporation of labeled lysine into connective tissue does not indicate a rapid turnover since in the collagen the loss of radioactivity is slow. However, metabolic activity of sponge-connective tissue would appear to be greater than that of tendon(12) since tagged hydroxylysine appeared in young collagen within 8 hours following injection of labeled lysine and only 30% of the radioactivity remained after 30 days. Specific activities of lysine in the acetic acid-soluble and -insoluble proteins of the 21-day old tissue (Table I) were greater than in the hot trichloroacetic acid-soluble collagen of the 17-day old tissue (Table II), and the difference is unquestionably related to metabolically active non-collagenous protein present in the 2 acetic acid fractions. The relatively rapid disappearance of radioactivity from the saline-soluble protein and the non-collagenous protein of the residue of connective tissue indicates a rapid turnover of these proteins of ground substance, the cellular protoplasm and the non-collagenous protein.

Incorporation of labeled lysine in the collagen fraction and a conversion of a portion to hydroxylysine are approximately the same for both sexes at 17 days of tissue age with, perhaps, the tissue of the male being somewhat more active than the female. Collagen of the 50-day old tissue from the female rat has approximately 50% of specific activities of lysine and hydroxylysine found in collagen from the 17-day old tissue. In the collagen iso-

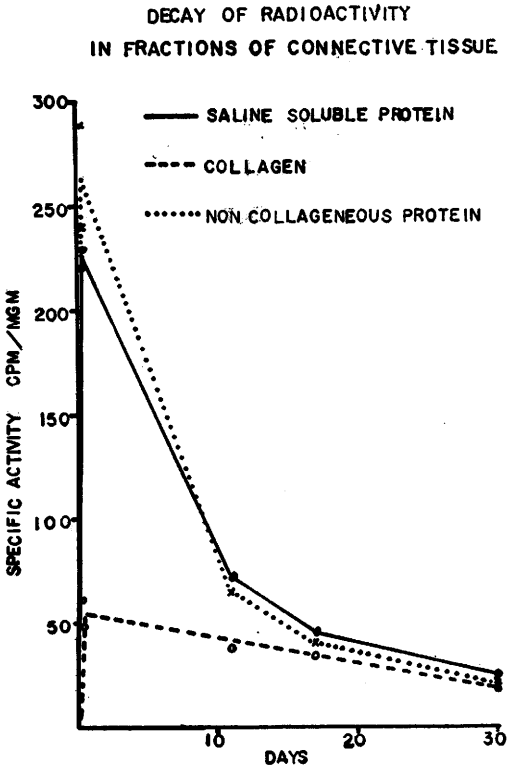

FIG. 2.

lated from the 90-day old tissue of the male rat there is a similar reduction. Specific activity of hydroxylysine in collagen of the 300-day old tissue of the male is 26% that of 17-day old tissue, while in the 300-day old tissue of the female, 55% of the 17-day tissue value was observed. This would seem to indicate that the female connective tissue has as active an hydroxylating mechanism at 300 days as found at 50 days of tissue age. It would appear that the tissue of the male has a reduction in rate of hydroxylation with tissue age. Yet it has been demonstrated that no increase in collagen content occurs after 21 days of tissue growth in the female rat, while the male tissue continues to accumulate collagen to 300 days of tissue age(6). This suggests that either rate of conversion of lysine to hydroxylysine is not an index of collagen formation or that the female tissue contains some substance which enhances breakdown of collagen at the same rate as its synthesis.

Summary. 1. C^{14}-labeled lysine administered intraperitoneally into the rat is incorporated within hours into the saline-soluble and -insoluble non-collagenous protein and collagen of biopsy-connective tissue. Radioactivity is confined to lysine in all fractions of connective tissue except for a portion which is converted to hydroxylysine in the collagen. 2. Following appearance of radioactivity in all fractions of connective tissue, decay occurs rapidly in saline-insoluble non-collagenous protein and saline-soluble protein and slowly in the collagen fraction. Thirty days after injection of labeled lysine approximately 10% of maximum activity remained in the saline protein fractions while 30% remained in collagen. 3. Incorporation of labeled lysine into the non-collagenous protein of connective tissue is not influenced by tissue age while its incorporation into the collagen appears to decrease with tissue age. 4. Specific activity of labeled hydroxylysine from a 300-day old tissue of a female rat was approximately twice that of a comparably aged tissue from a male rat.

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In vitro Incorporation and Hydroxylation of Lysine-2-C^{14} In Rat Biopsy-Connective Tissue.* (24096)

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Labeled lysine administered orally or intraperitoneally to the rat appears in the collagen fraction of connective tissue as lysine and hydroxylysine(1,2). The purpose of the present study was to determine the *in vitro* incorporation and hydroxylation of lysine-2-C^{14} in con-

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