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Biochem. J. (1961) **80**, 148

Metabolism of Collagen

APPEARANCE AND DISAPPEARANCE OF [¹⁴C]HYDROXYLYSINE IN RAT CONNECTIVE TISSUE

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The technique of labelling collagen with the radioisotope of a distinctive amino acid, hydroxylysine, simplifies the study of collagen metabolism. In collagen from adult animals this amino acid is derived from the hydroxylation of lysine after the 'parent' amino acid has been incorporated into a polypeptide (Sinex & Van Slyke, 1955, 1957; Sinex, Van Slyke & Christmas, 1959).

The metabolism of collagen has previously been studied by isotopically labelling a constituent amino acid. [¹⁴C]Glycine was used in investigations on the rabbit corium (Harkness, Marko, Muir & Neuberg, 1954) and by Jackson (1957) on the water- and salt-soluble and -insoluble fractions of the carageenin granuloma. However, glycine, though present in high concentrations in collagen,

does not specifically identify this fibrous protein. Green & Lowther (1959) studied collagen synthesis by following the conversion of [^{14}C]proline into [^{14}C]hydroxyproline in slices of carageenin granuloma. Problems germane to the isolation and purity of collagen are eliminated by the use of either the proline-hydroxyproline or the lysine-hydroxylysine system.

This paper presents the results of an investigation into the metabolism of [^{14}C]hydroxylysine-labelled collagen in the developing tissue *in vivo* of adult rats of both sexes.

METHODS

Connective tissue was obtained from adult male and female Sprague-Dawley rats (6-18 months of age) by the sponge-implant technique (Boucek & Noble, 1955). Three sponges were implanted aseptically along the dorsum in the loose subcutaneous fascia. All animals were maintained on Purina Laboratory Chow given *ad lib*.

The amount of [^{14}C]hydroxylysine in the sponge implants of 8, 12, and 20 days was determined. Seventy animals of each sex were treated, and the sponge tissues were removed at specified intervals (0.5, 1.0, 2.0, 3.0, 4.5, 6.0, 8.0, 9.5, 12.0, 14.5 and 16.0 hr.) after a single intraperitoneal injection of DL-[2- ^{14}C]lysine monohydrochloride (Tracerlab Inc., Waltham, Mass., U.S.A.; 12.5 $\mu\text{C}/100$ g. body wt.; 2.8×10^6 counts/min. in 21 μmoles dissolved in 0.5 ml. of 0.15M-NaCl, pH 7.4).

Immediately after removal, the implant was freed of surrounding fascia and weighed. The tissue was then homogenized (VirTis Macromhomogenizer, The VirTis Co. Inc., Yonkers, New York) in 7 ml. of cold 0.45M-NaCl (pH 7.4) for 0.5 min. at minimum and maximum speeds, transferred to a centrifuge tube and made to a volume of 25 ml. Centrifuging at low speed separated the fibres, and deoxyribonucleic acid (DNA) was determined on 0.5 ml. of the supernatant solution (Ceriotti, 1952). The mixture from the centrifuge tube was then placed in Lusteroid tubes and shaken for 24 hr. After centrifuging for 1 hr. at 55 000g (Spinco L preparative ultracentrifuge), the salt extract was removed. Three additional 24 hr. extractions with twice the tissue volume of 0.45M-NaCl (pH 7.4) were made (Gross, 1958). The combined salt extracts and the residue were dialysed against redistilled water until free of salt. All extraction and dialysis procedures were performed at 4° and in the presence of octan-2-ol. The two tissue fractions were dried, and collagen was extracted with hot (90°) trichloroacetic acid (Fitch, Harkness & Harkness, 1955).

Tissues from 25 animals of each sex were not subjected to salt extraction but were minced and extracted directly with trichloroacetic acid.

The trichloroacetic acid extracts were dried and subsequently hydrolysed for 16 hr. in 6N-HCl at 110° in sealed tubes. A portion of the hydrolysed sample was used for hydroxyproline determination (Neuman & Logan, 1950) and column chromatography after it had been dried three times to remove HCl and neutralized to pH 7.0.

Separation of the basic amino acids was effected by elution from a Dowex 50 X 8 (200-400 mesh) column by the procedure of Moore & Stein (1951). The neutral and

acidic acids were eluted with citrate buffer (pH 5.0) and the three basic amino acids, histidine, hydroxylysine and lysine, with phosphate buffer (pH 6.8). The phosphate buffer was collected in fractions (2 ml.) at the rate of 6 ml./hr. A portion (1 ml.) of each eluate fraction was dried on an aluminium planchet for measurement of radioactivity, and the amino acid concentration of the remaining 1 ml. was determined by the ninhydrin technique (Moore & Stein, 1954). The planchets were counted in a gas-flow counter with a thin-window Geiger-Müller tube. Corrections were made for background radioactivity and for the counting efficiency of the instrument. The thickness of the sample was such that the count obtained did not require a correction for self-absorption. The standard error calculated for the number of counts accumulated was 3.2-7.1% (Boucek, Noble & Woessner, 1959a).

The collagen content of 7-, 8-, 12- and 20-day implants from 139 animals was calculated from the amount of hydroxyproline in the tissue, with the conventional conversion factor of 7.46 (Neuman & Logan, 1950).

Blood samples were taken by cardiac puncture at intervals starting at 15 min. after the intraperitoneal injection. The radioactivity of the deproteinized serum (counts/min./ml.) was converted into lysine on the basis of the determined specific activity of the injected [^{14}C]lysine.

Calculations. Radioactivity of hydroxylysine was expressed in terms of either its specific activity (counts/min./mg.), its relation to cells (counts/min./mg. of DNA) or its content (counts/min./g. of sponge). The amounts of hydroxyproline (collagen) and DNA in the tissue were calculated in relation to the dry weight of the implanted sponge. The effect of variations in water content or non-collagenous substances of the tissue upon the data was thus eliminated.

DNA content (mg./g. of sponge) of 6-20-day tissues from 46 female and 32 male rats was plotted against tissue age, and the 'best-fit' lines were determined by the method of least squares. The value for any tissue age was obtained from the derived line.

RESULTS AND DISCUSSION

After the implantation of the sponge into the loose subcutaneous tissue, the interior becomes filled with blood elements, including leucocytes. It has been observed in histological sections that the leucocytes found during the first 7 days are ultimately replaced by fibroblasts (Boucek, 1959). The initial amount of DNA in the implant decreases until the sixth day (Boucek, Noble & Woessner, 1959b). This finding agrees with the histological observations of a decrease in leucocytes during the early period. From the sixth to the twentieth day the content of DNA increased linearly (Fig. 1), the amount in the female being greater than in the male. The cellular population during this period has been shown histologically to be made up principally of invading fibroblasts.

At present, the factors which stimulate the pre-existing fibrocytes to proliferate at the site of an injury are not fully understood. It has been observed, however, that even during the first

3 days after implantation, the concentration of DNA was greater in the female. This initial nucleic acid concentration was due to leucocytes and, accepting the premise that the products of leucocytes stimulate the production of fibroblasts (Carrel, 1922), the sex difference in the concentra-

tion of DNA in the 6-20-day tissue may be explained in part by the initial leucocytic response.

Appearance of [¹⁴C]lysine in the serum and of [¹⁴C]hydroxylysine in the tissue. The maximum observed activity of the serum occurred 15 min. after the injection and the value was unaltered 15 min.

Fig. 1. Deoxyribonucleic acid (DNA) content in 6-20-day connective tissue of adult rat: (a) female; (b) male. To obtain similar variance about the regression lines, square-root transformation was applied. No significant sex difference in variation was observed. Analysis of covariance indicated that the difference of the elevation of the lines, best estimated as 3.8 mg. of DNA/g. of sponge, was significant ($P < 0.001$).

Table 1. *Appearance of [¹⁴C]lysine in serum and of [¹⁴C]hydroxylysine in tissue*

Numbers of animals are shown in parentheses in the second column. Means and standard deviations of the serum radioactivities are given.

Time after injection (min.)	Serum (counts/min./ml.)	Tissue fraction	
		Salt-soluble (counts/min./mg. of hydroxylysine)	Salt-insoluble
15	50 398 ± 2 864 (4)	—	—
30	48 278 ± 12 102 (11)	4 854 5 403 5 657 5 632	121 57 55 —
60	25 260 ± 12 430 (9)	9 323 6 494 7 093 9 593	135 143 155 77 143
90	—	2 621 11 834 6 088	280 375 198 171
180	20 861 11 315 9 555 7 038	10 843 6 534 3 240 —	749 678 267 770 772

later (Table 1). The activity then decreased rapidly to a level which remained constant from the third to the eighth hour. Sex did not influence this response.

The appearance of [^{14}C]hydroxylysine in tissue from the 12-day implants of both sexes was measured during the 16 hr. after the intraperitoneal injection. Specific-activity data for the two sexes were combined because the amounts of collagen and labelled hydroxylysine were generally similar at this tissue age. Labelled hydroxylysine in a non-dialysable form appeared in the salt-soluble fraction within 30 min.; the specific activity of the hydroxylysine in this fraction continued to increase for a further 30 min. (a time when the specific activity of the serum was already declining), after which there was considerable variability (Table 1). A decline of the specific activity in the salt-soluble fraction occurred after the third hour, undoubtedly reflecting the diminished concentration of the serum [^{14}C]lysine.

As early as 30 min. after injection, a small amount of labelled hydroxylysine appeared in the salt-insoluble fraction. However, 60 min. after injection, a rapid accumulation of the labelled hydroxyamino acid began, at a rate of 258 counts/min./mg./hr. After the third hour, the rate slowed, due in part to the decline in the activity of the salt-soluble fraction.

Sex difference in the appearance of [^{14}C]hydroxylysine. The 1-3 hr. rate of synthesis of [^{14}C]hydroxylysine collagen expressed on a cellular basis (counts/min./mg. of DNA/hr.) was usually greater in the male (Fig. 2). Similar rates occurred only in the 12-day tissue. These results indicated that the cells from the male generally synthesize collagen more rapidly.

That such a sex difference has validity is established by the following facts: (1) The serum supply of free labelled lysine during the 1-3 hr. period was the same in both sexes, approx. 5.4 mg./100 ml. at the peak concentration (obtained by the procedure described in the Methods section). This concentration alone is more than twice the reported value for free serum lysine of the rat (Wu, 1954). (2) The vascular supply to the tissues appears to be similar on histological examination. An indirect measurement of vascularity is provided by haemoglobin determinations on tissue homogenates by Woessner in our laboratories (Flink & Watson, 1942). The haemoglobin value increased with tissue age, but was similar in both sexes. (3) The concentration of hydroxylysine in collagen from the sponge tissue of the two sexes was similar. Mature collagen is characterized by an hydroxylysine concentration of 1.3 g./100 g. (Bowes & Kenten, 1948), although younger forms of collagen, as reported by Van Slyke & Sinex (1958), have a

lower concentration (0.7 g./100 g.). In the 8-day sponge tissue of both sexes, a concentration of 0.65 g./100 g. was found in the trichloroacetic acid extracts of the insoluble fraction. At the twelfth and twentieth days the hydroxylysine of the collagen in this fraction increased to 0.8 g./100 g. (4) The ratio, hydroxylysine:hydroxyproline, in the salt-soluble and -insoluble collagen was not different for the two sexes.

The sex difference in the rates of synthesis was also evident in the 12 hr. kinetic study of the appearance of labelled hydroxylysine in young connective tissue. With this technique of intraperitoneal administration of [^{14}C]lysine, the labelled 8- and 12-day collagen of both sexes was found to reach maximum value between the eighth and the twelfth hours. A maximum value did not occur until 16 hr. in the 20-day tissues (Table 2). However, it was not possible to derive a meaningful kinetic expression for the appearance of [^{14}C]hydroxylysine beyond the third hour. Factors such as the decline in [^{14}C]lysine activity of the serum, the decrease in the concentration of salt-soluble [^{14}C]hydroxylysine and the process of collagen degradation contribute to the departure of the data from the linear expression of the 1-3 hr. period.

It was apparent from the peak concentration of the isotope that the 8-day tissue from the male had an extraordinary capacity for retention of a large amount of [^{14}C]hydroxylysine (Table 2). The 8-day tissue from the female contained fibroblasts with a lower rate of synthesis but the cells were more numerous. The [^{14}C]hydroxylysine of the 8-12 hr. peak therefore should have been greater than or equal to that of the male. However, the value from the male tissue appeared to be greater ($P < 0.05$), although the variability was great at this period. Obviously, some of the factors mentioned above, probably the process of collagen degradation, are

Table 2. [^{14}C]Hydroxylysine in young connective tissue

Numbers of animals are shown in parentheses. Means and standard deviations of the tissue radioactivities are given.

Time after injection (hr.) ...	Age of tissue (days)	Adult rat (counts/min./g. of sponge)	
		8-12	16
Male	8	2432 $\pm$ 813 (5)	1564 $\pm$ 682 (6)
	12	2008 $\pm$ 600 (8)	1665 $\pm$ 754 (12)
	20	1043 $\pm$ 195 (3)	1726 $\pm$ 426 (5)
Female	8	1352 $\pm$ 529 (5)	1151 $\pm$ 306 (6)
	12	2641 $\pm$ 253 (6)	1103 $\pm$ 202 (10)
	20	904 (2)	1003 $\pm$ 199 (5)

more active in the tissue from the female at this age. The resultant of collagen synthesis minus degradation is the net gain, and at 8 days a sex difference occurred. The collagen concentration (hydroxyproline conversion) was 113 ± 70 mg./g. of sponge in eight tissues from the male whereas ten tissues from the female had 57 ± 33 mg./g. of sponge ($P < 0.05$).

The peak quantity of [^{14}C]hydroxylysine of the 12-day tissue was greater in the female ($P < 0.05$, Table 2). Since the rates of synthesis were similar until this age, this larger quantity is undoubtedly attributable to the more numerous cells in the female.

Disappearance of [^{14}C]hydroxylysine. Only a part of the synthesized [^{14}C]hydroxylysine remained in the 8- and 12-day tissues. That this new collagen had a high rate of turnover was clearly indicated by the rapid disappearance of labelled hydroxylysine between the peak value and the sixteenth hour (Table 2). By the twentieth day this highly labile collagen was not detected.

Whereas collagen synthesis could be related to cellular activity, its disappearance could not. For example, no relationship was apparent between the 16 hr. value of [^{14}C]hydroxylysine collagen and the concentration of DNA. At the three tissue ages of the developing connective tissue of each sex, the 16 hr. concentrations of labelled collagen were similar in spite of an increasing concentration of DNA.

This similarity of the 16 hr. values strongly suggests the formation of constant amounts of collagen resistant to degradation and presumably in a crystalline state. This form of collagen was stable, remaining in the tissues for weeks.

The production of large amounts of collagen in the early stages of fibrosis probably lends stability to the initial gel formation of wound healing. In addition, this young collagen probably exists in a less well-organized state than does the highly crystalline mature collagen. The more random state of young collagen would undoubtedly enhance its susceptibility to degradation.

The finding of collagens with dissimilar metabolic properties was not surprising, for Harkness & Moralee (1956) have found uterine collagen to be rapidly metabolized. Jackson (1957) has reported on the extremely active synthesis and degradation of collagen in the carageenin granuloma.

The process of collagen degradation is not well understood. In the post-parturient uterus of the rat, collagen rapidly decreases within 2-3 days. Woessner (1959) reports the breakdown of collagen to the amino acid level in the post-parturient uterus, indicating the presence of active proteolytic enzymes. He has also noted the presence of large concentrations of proteases and peptidases in the

connective tissue of the implant (Woessner & Boucek, 1959). Older collagen, such as that of the corium, 'turns over' quite slowly, having almost an infinite half-life (Neuberger & Slack, 1953). However, the complete mechanism of collagen breakdown remains to be determined.

Sex differences in the disappearance of [^{14}C]hydroxylysine. In the 8- and 12-day tissues of both sexes, when large amounts of collagen were being synthesized, the disappearance of [^{14}C]hydroxylysine was rapid. The gradual accumulation of labelled collagen for 16 hr. in the 20-day tissue to a value approximating that remaining in the young tissue after active degradation indicates that the older tissue synthesized a collagen which probably polymerized directly into a crystalline form.

One consistent sex difference occurred in the 16 hr. values (counts/min./g. of sponge). The mean for 23 male tissues 16 hr. after the injection was 1600 counts/min./g. of sponge whereas the mean value for 21 female tissues was 1100 ($P < 0.001$). Conditions apparently exist in the male tissue which favour the formation of crystalline, more resistant collagen.

Factors which influence the crystalline state of collagen are unknown. Banfield (1952, 1956) reported a quantitative decrease in acid-soluble collagen and a decrease in the swelling of human Achilles tendon, and Kohn & Rollerson (1958) noted a decrease in acid-solubility of diaphragm-tendon collagen as a function of age. In 120-day human sponge-biopsy connective tissue from young, middle-aged and old donors, a difference in swelling with acetic acid occurred (Elden, Noble & Boucek, 1959). A host effect on this process was suggested, and these results were interpreted to indicate an increase in the degree of cross-linking in collagen with advancing age. The nature of these added cross-links is unknown, but their additions produce a greater crystallinity, which would resist swelling, acid-solubilization and digestion with collagenase (Keech, 1954). Presumably, it is the state of crystallinity in collagen which affects the tissue degradation of [^{14}C]hydroxylysine and the amount of resistant collagen (16 hr. value).

Collagen accumulation. After the insertion of the implant, collagen accumulates at a decreasing rate in the newly formed connective tissue of the rat. Previously reported data indicated that collagen accumulation in the female reached a maximum value of 246 mg./g. of sponge by the fortieth day. In the male, collagen accumulation continued beyond 100 days to a value of 344 mg./g. of sponge (Kao, Boucek & Noble, 1957; Noble, Woessner, Kao & Boucek, 1958; Boucek *et al.* 1959a, b).

Seven days after implantation, the concentration of collagen varied considerably: 18 ± 13 mg./g. of sponge (mean value for tissues from 10 female

Fig. 2. Collagen synthesis in connective tissue of the rat. At each tissue age, rates were calculated from at least four points during the 1-3-hr. period after injection of [^{14}C]lysine: (a) 8-day tissue; (b) 12-day tissue; (c) 20-day tissue; ● (—), female; ▲ (---), male.

and 14 male rats). The kinetic studies of [^{14}C]hydroxylysine-collagen synthesis indicated a rapid rate at 8 days in both sexes. However, the net gain of collagen by the eighth day appeared to be less in the tissue from the female. This suggests a greater lability of collagen in the female and the formation of a larger proportion of the stable form by the male. A rapid gain in collagen occurred in the female by the twelfth day. This may be correlated with the enhanced rate of synthesis by the 12-day cells in the females (Fig. 2) and with a less rapid breakdown than in the 8-day implants. The mean concentration after 12 days was 133 ± 28 mg./g. of sponge in 33 female and 149 ± 34 in 34 male rats. By 20 days, the mean concentration in 13 tissues from the female was 164 ± 27 , and in 17 tissues from the male the concentration was 195 ± 64 .

SUMMARY

1. Collagen metabolism in developing connective tissue of the adult rat was studied by the techniques of sponge-implantation and the specific labelling of collagen with [^{14}C]hydroxylysine. This labelling was introduced by intraperitoneal administration of [^{14}C]lysine, which reached its maximum observed serum concentration within 15 min. The labelled lysine was hydroxylated in the newly synthesized peptide destined for collagen.

2. Young connective tissue was characterized by the synthesis of a large amount of labile collagen. Stable collagen was formed either as a part of the labile-collagen synthesis or independently. This stable form was constant in amount at 8, 12 and 20 days' tissue age and remained in the tissue for weeks. The extremely labile form of collagen was synthesized only during the early period of con-

nective-tissue formation. At 20 days, synthesis of this form of collagen was not detected.

3. The metabolism of collagen was influenced by the sex of the host. Isotopic studies indicated a more active synthesis of collagen and a formation of larger amounts of stable collagen by the cells of the male. These characteristics should effect a greater accumulation of collagen in the tissue from the male.

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The Hydrolysis of Phenyl Phosphate by Mouse-Liver Acid Phosphatase

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To determine acid-phosphatase activities of liver homogenates, with phenyl phosphate as substrate, previous workers have usually adopted methods similar to the Robinson, Gutman & Gutman (1939) adaptation of the King & Armstrong (1934) method for serum phosphatase (see, for example, Greenstein, 1942; Kochakian & Fox, 1944; Abul-Fadl & King, 1949; Goodlad & Mills, 1957). However, results obtained by Tsuboi (1952), in studies of the hydrolysis of phenyl phosphate by mouse-liver acid phosphatase, have indicated that the assay conditions of the Robinson *et al.* (1939) method, and of similar methods, allow only partial expression of the capacity of liver homogenate to hydrolyse phenyl phosphate in acid media.

In the present work, a re-examination has been made of the hydrolysis of phenyl phosphate by mouse-liver acid phosphatase and, although the results have differed from those of Tsuboi (1952), it has been confirmed that the Robinson *et al.* (1939) procedure is unsuitable for quantitative measurement of liver acid-phosphatase activity. From the results of these studies a simple and rapid method for quantitative measurement of mouse-liver acid-phosphatase activity with phenyl phosphate has been developed.

MATERIALS AND METHODS

Experimental animals were normal, well-fed, adult male mice of mixed laboratory strains. Chemicals were of A.R. grade whenever possible. Spectrophotometric measure-

ments were made on a Unicam spectrophotometer (SP. 500) and pH measurements on a model C pH electrometer (N. L. Jones, Melbourne). Enzyme assays and homogenate incubation were made at 37° with shaking racks fitted to a Braun model V rotary Warburg bath.

Phenol solution. A stock phenol soln. (1 g./l.), prepared by dissolving distilled phenol in *N*-HCl, was standardized by iodometric titration (Cameron & White, 1940). $E_{290} - E_{320}$ and $E_{295} - E_{320}$ values, for *m*-phenol in *N*-NaOH, were 2.46 and 1.89 respectively. Corresponding values of 2.45 and 1.92 were reported by Brawerman & Chargaff (1954).

Phenyl phosphate. Commercial disodium phenyl phosphate was purified by the method of Tsuboi (1952). Total phosphorus of product, 14.3%; theoretical for $\text{Na}_2\text{C}_6\text{H}_5\text{PO}_4$, 14.2%.

Ethylenediaminetetra-acetate. The commercial disodium salt of ethylenediaminetetra-acetic acid (EDTA) was purified by the method of Blaedel & Knight (1954).

Sulphydryl compounds. *p*-Chloromercuribenzoic acid was prepared according to Whitmore & Woodward (1941). Phenyl mercuric acetate was obtained from British Drug Houses Ltd., and *o*-iodosobenzoic acid from L. Light and Co. Ltd. Aqueous solutions of these compounds were prepared with minimal NaOH.

Fresh solutions of L-cysteine hydrochloride (British Drug Houses Ltd.) were adjusted to pH 5.9 before use.

Preparation of liver homogenates. Preliminary studies of the intracellular distribution of mouse-liver acid phosphatase, with phenyl phosphate as substrate, indicated that mitochondria contained unreactive enzyme (equivalent to approx. 40% of the total enzyme activity) and that suspension of the mitochondria in water led to activation of this bound enzyme. These results were in general agreement with those obtained by Berthet & de Duve (1951) in studies of the hydrolysis of β -glycerophosphate by rat-liver acid