Synthesis and Turnover of Collagen Precursors in Rabbit Skin

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(Received 24 June 1966)

1. The rate of synthesis of [^{14}C]hydroxyproline by rabbit skin was studied *in vitro* and *in vivo*. 2. The soluble collagen fractions were shown to have a very rapid turnover. The 0.15M-sodium chloride-extractable collagen showed t_1 values of 1.2 hr. *in vitro* and 12 hr. *in vivo*. The 0.5M-sodium chloride-extractable collagen exhibited a t_1 value of 20 hr. *in vivo*. 3. Under the conditions used it was not possible to obtain radioactive insoluble collagen *in vitro*. 4. A significant amount of soluble collagen is lost before it becomes insoluble. 5. These observations may help to explain why large amounts of peptide-bound hydroxyproline appear in the urine during periods of rapid collagen synthesis.

Collagen is synthesized by fibroblasts as a soluble precursor or tropocollagen molecule that undergoes a process of fibrillogenesis and aggregation while in an extracellular environment. Neutral-salt-soluble collagen is believed to be the primary precursor of mature insoluble collagen. It can be denatured into three peptide chains of equal molecular weights, the α -components (Orekhovich, Shpikiter, Mazurov & Kumna, 1960; Piez, Eigner & Lewis, 1963). The acid-soluble collagens represent a more mature stage and are characterized by a more stable dimerization as well as a small amount of trimerization of the α -peptide chains (Orekhovich & Shpikiter, 1958a). The acid-soluble components therefore represent an intermediate stage towards the mature insoluble collagens. Orekhovich & Shpikiter (1958b) found that [^{14}C]glycine was much more rapidly incorporated into a citrate-soluble collagen than into the insoluble residue. Harkness, Marko, Muir & Neuberger (1954) observed that radioactive collagen could be isolated even earlier if the tissue was extracted with a slightly alkaline buffer (pH 9.0) and that this fraction had a shorter half-life than the citrate-soluble collagen. Subsequent to these findings investigators have used 0.15M-sodium chloride as such or buffered with phosphate to pH 7.5 to extract the more recently synthesized collagenous material (Jackson, 1957).

The feeding of radioactive amino acids to young actively growing animals results in the deposition of collagen of an initial high specific activity (Neuberger, Perrone & Slack, 1951). This specific activity has been shown to decrease with age. This decrease can be accounted for by isotope dilution occurring as a result of accretion of newly synthe-

ized non-radioactive material associated with growth. However, the total radioactivity of the body collagen did not diminish greatly, indicating a very low turnover rate. The specific activity of skin collagen after the injection of [^{14}C]glycine has been reported to be greater in young animals than in older ones (Neuberger & Slack, 1953; Nimni & Bavetta, 1961), reflecting a fall in the rate of synthesis with age.

A turnover time of more than 150 days for the insoluble fraction of skin collagen and 25 days for the citrate-soluble fraction has been reported in experiments with [^{14}C]proline (Gerber, Gerber & Altman, 1960). This relative inertness of insoluble collagen is further supported by the findings of Popenoe & Van Slyke (1962) on [^{14}C]lysine. On the other hand, Kao, Hilker & McGavack (1961), also using [^{14}C]lysine, obtained t_1 values of 28 days for the insoluble collagen and 17 days for the soluble fraction. Nimni & Bavetta (1964), using the biopsy technique, have reported the changes in radioactivity of collagen in the skin of rats observed over a period of 115 days after a single tracer dose of [^{14}C]glycine. The fall in specific activity of the insoluble collagen, after correction for isotope dilution resulting from growth, did not reflect the presence of a single homogeneous component. This lack of apparent homogeneity may reflect a progressive decrease of solubility with age. A t_1 value of 28 days was evident for this fraction during the period of rapid growth whereas a value close to 300 days could be obtained by extrapolation towards the end of the experiment. On the other hand, the specific activity of the soluble collagen fractions decayed almost exponentially, indicating that their rate of turnover was not age-dependent.

Nevertheless, satisfactory values for determining the rate of transformation of the collagen precursors into insoluble fibre and the efficiency of such a system could not be adequately obtained from such a long-term experiment owing to the inherent variables present. In the present series of experiments we have made attempts to evaluate further the process of collagen aggregation based on the progression of the labelled collagen precursor through the different fractions leading to eventual insolubility, employing systems both *in vitro* and *in vivo*.

EXPERIMENTAL

Animals. Four-week-old New Zealand white male rabbits weighing 550–650 g. were used in all experiments. We have found this age to be optimum for a study of this nature, since there are present in the skin at this time significant amounts of soluble and insoluble collagen in an active metabolic state (Nimni, de Guia & Bavetta, 1965).

Studies in vitro. Freshly excised rabbit-skin specimens were weighed and immediately placed in beakers containing 15 ml. of Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing 29.6 μ moles of glucose/ml. and 3 μ C of [U- 14 C]proline. They were incubated for 30 min. in a Dubnoff shaker under an $O_2 + CO_2$ (95:5) atmosphere at 37°. After this period of preincubation they were washed twice in 0.15 M-NaCl and placed in a Krebs–Ringer bicarbonate medium containing unlabelled proline (20 μ M). Incubation was carried on for different time-intervals over a 6 hr. period. Each skin specimen was then removed from the medium and rapidly frozen at –75°. Duplicate samples were tested at each time-interval. For analysis they were minced in the cold (4°) and homogenized for 45 min. with 10 vol. of cold 0.15 M-NaCl in a VirTis homogenizer. They were shaken for 18 hr. at 4° and centrifuged at 30000g for 1 hr., and the supernatants were dialysed first against running tap water for 20 hr. and then against distilled water for 4 hr. The residue from the previous centrifugation was re-extracted subsequently with 0.5 M-NaCl, followed by 0.5 M-citrate buffer, pH 3.6. The residue was converted into gelatin by autoclaving. All fractions were dialysed in the cold. Samples were hydrolysed in 6 N-HCl by heating for 2.5 hr. at 121° in Teflon-lined screw-cap test tubes. The collagen content was estimated by measuring hydroxyproline (Woessner, 1961). Samples were taken for the isolation of proline and hydroxyproline and pooled in pairs for determination of their specific activities. For this purpose the *N*-nitroso imino acid derivatives were prepared by the method of Myhill & Jackson (1963). The nitroso imino acids were dissolved in 1.0 ml. of 0.2 N-citrate buffer, pH 2.2. They were then separated on a column (69 cm. $\times$ 0.9 cm.) containing Beckman spherical resin type AA-15 jacketed at 55°, with 0.2 N-citrate buffer, pH 3.28, as eluent. The Beckman amino acid analyser model 120B was used to automate this procedure (Bekhor, Mohseni, Nimni & Bavetta, 1965). The effluent was passed through a 1.0 ml. flow cell in a scintillation spectrometer at a flow rate of 1.0 ml./70 sec. Specific activity was calculated in terms of counts/min./ μ mole of imino acid. Recovery by this procedure was in the range 95–100%. Efficiency of counting was

about 70%. The results represented graphically correspond to the specific activity of the hydroxyproline in each fraction, as well as the total radioactive imino acid in each fraction present in 1 g. wet wt. of skin. This latter form of presenting the results allows one to account for differences in pool size and is more useful for understanding the rate at which turnover is occurring.

Studies in vivo. Rabbits were injected intravenously with 17 μ C of [U- 14 C]proline (specific activity 186 mc/m-mole) in 0.15 M-NaCl/kg. body wt. One rabbit was killed 5 hr. later, at which time the rest of the animals were slowly infused with 300 mg. of proline intravenously and 450 mg. of proline intragastrically to dilute the specific activity of the circulating and tissue [14 C]proline. The remaining rabbits, two for every time-interval, were killed 10, 24, 48 and 120 hr. after the [14 C]proline injection. In addition, one animal was killed 2.5 hr. after the [14 C]proline injection to detect early labelling. Blood samples for monitoring circulating radioactivity were taken at short intervals after the [14 C]proline injection and at the time of death. Radioactivity was determined on a sample of a deproteinized specimen. Samples of dorsal skin were removed, cleaned from adhering adipose and muscle tissue, cut into small pieces, weighed and immediately homogenized with 10 vol. of cold 0.15 M-NaCl in a VirTis homogenizer. From there on they were processed as indicated above and the specific activities of proline and hydroxyproline determined in each fraction. Only the results for hydroxyproline are presented as they showed good agreement in all instances with those of proline, except for the 0.15 M-NaCl fraction where there were slight discrepancies owing to contamination with non-collagenous materials. This uncertainty is eliminated when the hydroxyproline values are used, since this imino acid is present exclusively in collagen.

RESULTS

The proline and hydroxyproline contents as well as the radioactivity present in the 0.15 M- and 0.5 M-sodium chloride fractions extracted from 1 g. of skin specimens after incubation for different times are shown in Table 1. During the first 0.5 hr. the skin specimens were exposed to [14 C]proline, by which time no labelling of hydroxyproline had occurred. This delay in hydroxylation has been reported previously in the chick embryo (Peterkofsky & Udenfriend, 1963) and is presumably the time required for microsomal proline to be hydroxylated (Prockop & Juva, 1965). Hydroxyproline radioactivity rose rapidly in the 0.15 M-sodium chloride-extractable fraction after the tissue was transferred to the non-radioactive medium, reflecting the appearance of newly synthesized collagen (1 hr. total incubation). The more aggregated material that was not extracted by 0.15 M-sodium chloride but dissolved in 0.5 M-sodium chloride showed a much slower but continuous rise. After about 2.5 hr. of incubation a steady state developed in the 0.15 M-sodium chloride fraction that may reflect a decrease in the biological activity of the system. We could not detect radioactive proline or hydroxy-

Table 1. *Proline and hydroxyproline contents and radioactivity recovered from the 0.15M- and 0.5M-sodium chloride fractions in 1g. of rabbit skin after different time-intervals*

Samples were preincubated for 30min. with $3\mu\text{C}$ of $[\text{U-}^{14}\text{C}]$ proline and then transferred to non-radioactive medium. Details are given in the text.

Incubation time (hr.)	0.15M-NaCl fraction				0.5M-NaCl fraction			
	Proline		Hydroxyproline		Proline		Hydroxyproline	
	(μmoles)	(counts/min.)	(μmoles)	(counts/min.)	(μmoles)	(counts/min.)	(μmoles)	(counts/min.)
0.5	5.65	85	1.05	3	4.18	50	3.30	0
1.0	7.09	1363	1.44	678	2.82	192	2.08	161
1.5	6.67	1634	1.37	496	3.54	188	2.10	105
2.5	7.39	1108	1.57	232	3.28	194	2.13	196
4.5	11.55	2277	3.73	553	3.37	283	2.02	253
6.5	7.38	1381	2.03	266	2.37	263	2.18	285

Fig. 1. Specific activity of hydroxyproline isolated from different skin fractions at different time-intervals after a 0.5 hr. preincubation period with $[\text{U-}^{14}\text{C}]$ proline. All the radioactive hydroxyproline present was extracted in the 0.15M-NaCl (□) and 0.5M-NaCl (▲) fractions.

proline in either the 0.5M-citrate extract or the insoluble residue remaining in the skin from the above specimens; this indicated that fibrous insoluble collagen was not synthesized by this system under these conditions over the 6 hr. period. The specific activity values for hydroxyproline calculated from the results in Table 1 are plotted in Fig. 1. The specific activity of the 0.15M-sodium chloride fraction reached a peak after 1 hr. and began to fall thereafter, reaching a steady state after 2.5 hr. There is no evidence of a double peak under these circumstances, as had been reported in the experiments *in vivo* of Bentley & Jackson (1963). The 0.5M-sodium chloride extract showed a gradual rise of specific activity, reflecting a product-dependence effect and a much lower rate of turnover.

Our inability to obtain labelled citrate-soluble or

Fig. 2. Changes in serum radioactivity after the intravenous injection of $17\mu\text{C}$ of $[\text{U-}^{14}\text{C}]$ proline/kg. body wt. to 4-week-old rabbits. At 5 hr. after the ^{14}C proline injection the animals were injected with 2ml. of 15% (w/v) proline solution and 1 hr. later fed with 3ml. of the same solution, to dilute the specific activity of the radioactive proline.

insoluble collagen under the conditions of the system *in vitro* made it necessary to employ a technique *in vivo* to extend the experimental period to 120 hr.

The changes in total serum radioactivity after the ^{14}C proline injection and the subsequent administration of non-radioactive proline are shown in Fig. 2. This curve illustrates the rapid disappearance of labelled material from the circulation.

The specific activity of the hydroxyproline isolated from the different collagen fractions at different time-intervals is shown in Fig. 3. The curve representing the most recently synthesized

Fig. 3. Specific activity of hydroxyproline present in the different collagen fractions isolated from skin at intervals after the injection of [U- ^{14}C]proline. □, 0.15M-NaCl fraction; ▲, 0.5M-NaCl fraction; ○, 0.5M-citrate (pH 3.6) fraction; ●, insoluble collagen.

collagen (0.15M-sodium chloride fraction) indicates that this fraction may have reached its maximum specific activity before the first sample was taken (2.5 hr.). It was shown *in vitro* to reach a maximum 1 hr. after the exposure of the skin to [^{14}C]proline. The slightly less soluble collagen, which is extracted by 0.5M-sodium chloride, reaches its maximum specific activity between 5 and 10 hr. after [^{14}C]proline administration. A similar finding is seen for the insoluble collagen, whereas the citrate-extractable material reached maximum hydroxyproline labelling between 24 and 48 hr.

The pool size has again to be considered to determine the efficiency of the collagen-synthesizing system and to account for the net displacement of the radioactive hydroxyproline through the different solubility fractions. The results, expressed as counts/min. of hydroxyproline in each fraction/g. wet wt. of skin, are summarized in Fig. 4.

Once the collagen has lost its earlier capacity to dissolve in 0.15M-sodium chloride, and requires a higher ionic strength to dissolve it (0.5M-sodium chloride fraction), it proceeds with more efficiency into the fractions of decreasing solubility. The maximum radioactivity in the 0.5M-sodium chloride fraction was reached after 5 hr. At this time the total radioactive hydroxyproline present in all four fractions isolated was 1580 counts/min./g. of tissue. Four days later the total radioactivity present in

Fig. 4. Total radioactive hydroxyproline present in 1 g. of skin. These values were calculated by multiplying the specific activity by the amount of collagen present in each fraction in 1 g. of skin and are meant to take into account the isotope dilution due to the magnitude of the pool size. □, 0.15M-NaCl fraction; ▲, 0.5M-NaCl fraction; ○, 0.5M-citrate (pH 3.6) fraction; ●, insoluble collagen.

skin, most of which can be found in the more inert fractions, was 1020 counts/min./g. of tissue. If the replacement rate of the insoluble collagen is ignored because of its slow turnover, it can be calculated that not more than 65% of the hydroxyproline present initially in the 0.5M-sodium chloride fraction will even reach the insoluble stage. This difference is too large to be accounted for by the simple process of growth during the short interval of this observation.

DISCUSSION

Our studies indicate that the conversion of soluble collagens into insoluble fibres is not a quantitative process. It appears that very significant amounts of the soluble collagen components are lost for the purpose of forming insoluble fibres. It has been shown that considerable amounts of peptide-bound hydroxyproline appear in the urine under conditions of rapid collagen synthesis, such as that seen in growth (reviewed by Smiley & Ziff, 1964). Since the excretion of peptide-bound hydroxyproline is greater in children than in adults, it was suggested that the concentration of urinary hydroxyproline might be related to the size of the soluble collagen pool.

After giving [^{14}C]proline to young rats Lindstedt & Prockop (1961) found that the changes in the specific activity of urinary hydroxyproline indicated that it was being derived from at least three

different hydroxyproline pools. Two of the pools had t_1 values of 1 and 5 days and the third showed a t_1 value of over 100 days. These values are comparable with those in the literature for the t_1 values of different soluble and insoluble collagen fractions.

Our studies indicate that a very significant amount of the peptide-bound hydroxyproline extractable by 0.15M-sodium chloride, which has a very fast turnover (t_1 1.2hr. *in vitro*; t_1 12hr. *in vivo*), is not converted into the 0.5M-sodium chloride fraction and is lost for the purpose of forming insoluble fibres. It is not possible under our experimental conditions to estimate the efficiency of this conversion, but the loss of peptide-bound hydroxyproline is considerable. It is likely that it is this material, which is never converted into an insoluble collagen fibre, that will give rise to a major part of the peptide-bound hydroxyproline that is encountered in the urine. From our studies *in vivo* we can also observe that a significant proportion of the 0.5M-sodium chloride-soluble collagen is lost and made unavailable for fibre formation. From our results we estimated that approx. 35% of the 0.5M-sodium chloride collagen fraction will not aggregate into fibres and will eventually be shifted into some other pathway.

This research was supported by Grant no. DE-00626-08 and Training Grant no. 5-T1-DE-94-02 from the U.S. Public Health Service, Institute of Dental Research. L. A. B. is a recipient of Career Development Award from the U.S. Public Health Service, Institute of Dental Research.

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