EVIDENCE FOR AN ISOTOPIC STEADY STATE IN SOLUBLE COLLAGENS

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SUMMARY. The distribution of specific radioactivity was determined for soluble and insoluble collagens in guinea pigs multiply labeled with ^{3}H-L-proline 6 weeks prior to sacrifice. Multiple extractions of neutral salt-soluble (0.15-0.30 M NaCl) and citrate-soluble collagens demonstrated constant but unequal specific activities which indicated the achievement of an isotopic steady state. The specific activity of acid-soluble collagens is 3-5 times higher than salt-soluble collagen and almost equal to that of insoluble collagen. The data indicate that there is a large difference in biological age between salt-soluble collagen and acid-soluble collagens.

INTRODUCTION. The extractions of soluble collagens from labeled tissues have been done at different time intervals after the administration of radioisotope. Decreasing specific activities were observed in neutral-soluble and acid-soluble collagens during the first 8 hours to 7 days after labeling (Orekhovitch, 1952; Harkness *et al.*, 1954; Jackson and Bentley, 1960). However, examination of their results 21 to 40 days after labeling, revealed that after the initial extractions removed the neutral salt-soluble collagen, the specific activities of acid-soluble collagens remained relatively constant (Harkness *et al.*, 1954; Orekhovitch and Shpikiter, 1958; Lapiere *et al.*, 1966). The difference in constancy of specific activity with time after labeling suggested a re-evaluation of the isotopic heterogeneity of soluble collagens. This communication describes the specific activity of sequential extractions of salt-soluble and acid-soluble collagens, six weeks after multiple labeling of guinea pigs.

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METHODS. Actively growing female guinea pigs, weighing 242-251 g, received intraperitoneal injections of L-proline-3,4-^{3}H (0.25 μC/g body weight) twice per week for four weeks (total of 850 μC). Guinea pigs weighed 429-443 g at the time of the last injection of tritiated proline. Six weeks after the end of labeling, the guinea pigs (626-666 g) were sacrificed. Dorsal skins (5 g, wet weight) were clipped, cleaned from subcutaneous tissue and finely minced but not homogenized. Each skin was sequentially extracted with 100 ml of neutral salt solutions (0.15 M to 2.0 M,pH 7.4), 0.25 M or 0.5 M citrate, pH 3.6, and 0.25 M or 0.5 M acetic acid. Skins were extracted for 24 hr when two or more extractions were done with similar media, otherwise single extractions were done for 72 hr. Each skin was stirred by air-driven magnetic stirrers at 4° C. Toluene was added to each extraction medium to prevent bacterial growth. The extracted soluble collagens were separated from insoluble collagen by centrifugation at 15,000 X g for one hr, followed by filtration through glass wool. (Preliminary experiments had demonstrated that centrifugation at speeds from 7,000 to 105,000 X g did not affect the specific activity of salt-soluble collagens). The filtrate was dialyzed against several changes of 0.02 M Na_2HPO_4, and the resulting fibrous precipitate was separated by centrifugation. Soluble and insoluble collagens were hydrolyzed, and hydroxyproline was separated by column chromatography prior to chemical and isotopic analysis as previously described (Klein and Weiss, 1966).

RESULTS AND DISCUSSION. Figure 1 demonstrates that the specific activity of hydroxyproline from neutral salt-soluble collagens extracted by 0.15 M and 0.30 M NaCl was constant. After the first extraction the specific activity of citrate-soluble collagens was constant, but three times greater than neutral salt-soluble collagen and 80% that of insoluble collagen (Fig. 2). The sixth extraction contained 0.6 mg of hydroxyproline as compared to 8.5 mg in the first extraction of citrate-soluble collagen. The acetic acid-extractions, after the first two extractions, approach the specific activity of insoluble collagen. The constancy of specific activity within neutral salt-soluble

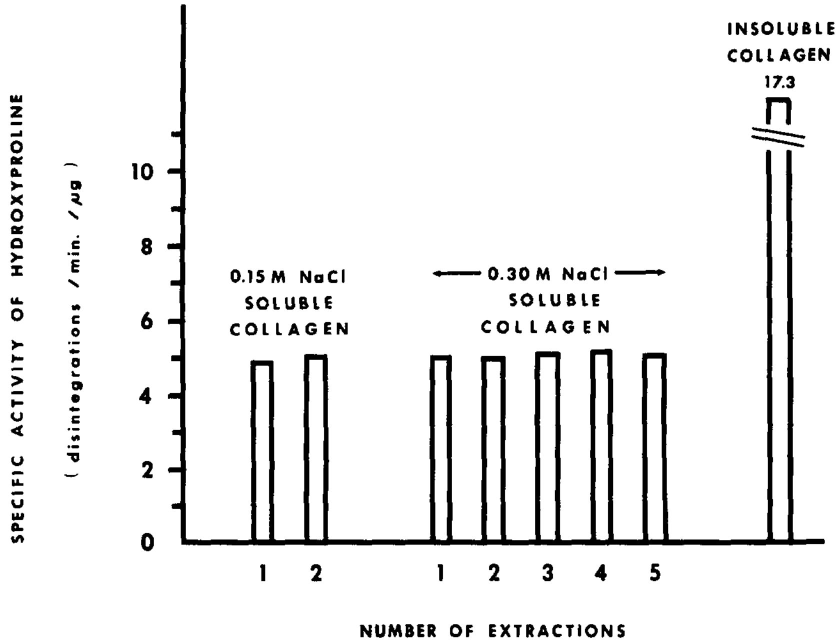


Figure 1. Specific activity of hydroxyproline from neutral salt-soluble collagens after daily sequential extractions of guinea pig skin. Each bar represents the mean of two experiments.

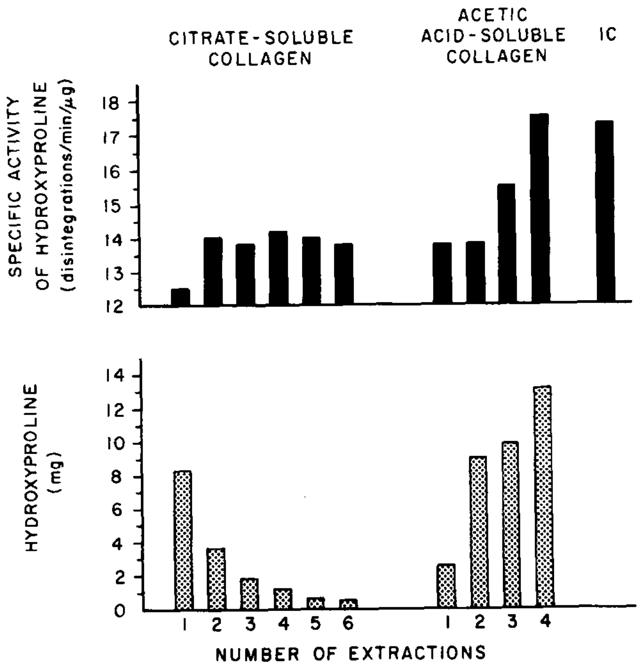


Figure 2. Amount and specific activity of hydroxyproline from citrate-soluble, acetic acid-soluble and insoluble (IC) collagens of guinea pig skin. 0.25 M Citrate buffer, pH 3.6 and 0.25 M acetic acid were used. Each bar represents the mean of two experiments.

and citrate-soluble collagen (excluding acetic acid-soluble collagen) indicates the achievement of an isotopic steady state, thus permitting an interpretation of specific activity (Tarver, 1954). Similar results have been observed in rats 6 weeks and 5 months (Klein, 1969), and 15 months (unpublished results) after labeling.

Prelabeled rats fed a diet containing 15% non-radioactive L-proline (Klein, 1969) markedly decreased the specific activity of neutral salt-soluble collagen to 40% of the controls, but not that of citrate-soluble collagen. These observations indicate that most of the radioactivity found in hydroxyproline of neutral salt-soluble collagen was due to ^{3}H-proline reutilization in *de novo* collagen synthesis rather than the conversion of insoluble collagen to neutral salt-soluble collagen (Jackson, 1957).

The reutilization of proline radioactivity under steady state conditions could not account for the high specific activity of hydroxyproline found in citrate-soluble, acetic acid-soluble and insoluble collagens. The latter results indicate that acid-soluble collagens were synthesized at the time of labeling, and suggest that citrate-soluble collagen is derived from old fibrous* collagen which has not been cross-linked intermolecularly. This interpretation is consistent with the destructive effect of acid buffers on fibrous collagen of calf skin as seen by the electron microscope (Gross *et al.*, 1955). Unlike the extraction end point of salt-soluble collagen (Gross *et al.*, 1955; Jackson and Bentley, 1960) and citrate-soluble collagen (Bowes *et al.*, 1955, and Fig. 2), the continuous release of acetic acid-soluble collagen (Fig. 2) with the highest specific activity suggests that acetic acid extracts insoluble collagen.

Table I shows the effect of extracting salt-soluble collagens sequentially with increasing concentrations of NaCl. The specific activity of salt-soluble collagens extracted with 0.15 M and 0.30 M NaCl was constant. A gradual increase in specific activity was observed after 0.45 M to 2.0 M NaCl

* A distinction is made between fibrous collagen that is easily extractible and fibrous collagen that is considered insoluble with present techniques.

TABLE I

SPECIFIC ACTIVITY OF SOLUBLE COLLAGENS FROM GUINEA PIG SKIN

Extraction media	Hydroxyproline (mg)	Specific activity of hydroxyproline (disintegrations/min/μg)
0.15M NaCl (3X)	2.60	5.7
0.30M NaCl (2X)	3.73	5.6
0.45M NaCl (2X)	4.74	7.5
1.00M NaCl (1X)*	0.72	8.7
2.00M NaCl (1X)*	0.16	8.8
0.50M citrate (2X) (pH 3.5)	19.00	17.3
0.50 acetic acid (3X)*	7.00	19.1
Insoluble	55.90	20.4

* Each extraction was done for 24 hours; number of extractions are given in parentheses.

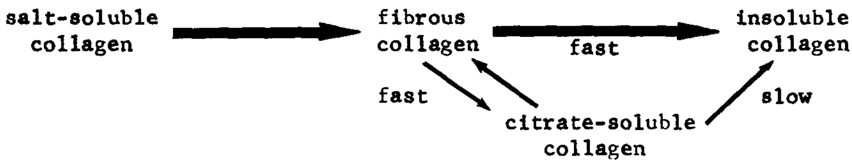
was used. The specific activity of 1.0 M and 2.0 M NaCl-soluble collagen was the same, and one-half that of citrate-soluble collagen.

The increasing specific activities of 0.45 M, 1.0 M and 2.0 M NaCl-soluble collagens over that observed in 0.15 M and 0.30 M NaCl-soluble collagen coincides with the first appearance of β-component in 0.45 M NaCl-soluble collagen, and with an increase in the amount of β-component extracted with 1 and 2 M NaCl (Mazurov and Orekhovich, 1961; Bakerman, 1964). Moreover, alkali-soluble (Mazurov and Orekhovich, 1961) or 0.15 M NaCl-soluble collagen (Bakerman, 1964) contain only α-chains, while β-chains are found in citrate-soluble and acetic-acid soluble collagens. Thus, the differential increase in specific activity of neutral salt-soluble collagens observed under steady state conditions suggests that salt-soluble collagens extracted with

0.45 M to 2 M NaCl are contaminated with acid-soluble collagen rather than a gradual maturation of α-chains to β-chains. Any mixing of neutral salt-soluble and acid-soluble collagen would give the heterogenous results (Mazurov and Orekhovich, 1961) that were observed isotopically. However, heterogeneity of α-chains due to metabolic modifications of lysine (Piez *et al.*, 1966) would not be observed under our isotopic conditions.

If all of the β-chains are coming from *de novo* synthesis, then α- and β-chains should have specific activities similar to that of salt-soluble collagen. A comparison of specific activities of α- and β-chains from salt-soluble and acid-soluble collagens are under investigation.

The isotopic data suggests the following relationship:



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