PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 151, 385-389 (1976)

The Intermolecular Cross-Links in Uterine Collagens of Guinea Pig, Pig, Cow, and Human Beings[1] (39217)

KUNG YING TANG KAO, WILLIAM E. HITT, AND JAMES G. LESLIE

Geriatrics Research Laboratory, VA Center, Martinsburg, West Virginia 25401

The importance of collagen as supporting tissue is attributed to its molecular alignment and its intermolecular cross-links. The reducible intermolecular cross-links have been stabilized with mild reducing agent, tritiated $NaBH_4$, isolated, and identified (1-7). They are lysine- or hydroxylysine-derived aldehydes reacting with themselves to form aldol condensation products or reacting with ϵ-amino group of another molecule of lysine or hydroxylysine to form a Schiff base. The aldol can further react with a molecule of hydroxylysine to form hydroxymerodesmosine (7), or with histidine to form aldolhistidine (5), or with both hydroxylysine and histidine to form histidinohydroxymerodesmosine (6). The occurrence and abundance of these cross-links vary with species, tissue, and age of the animals studied.

Previous work from our laboratory revealed that uterine collagen metabolizes more actively than collagens from other organs (8). In addition, uterine collagen increases rapidly during pregnancy and decreases rapidly after parturition (9, 10). Therefore, it is of interest to study the cross-links in uterine collagen.

Recently we have reported that the intermolecular cross-links in rat uterine collagen are found to be dehydrodihydroxylysinonorleucine and dehydrohydroxylysinonorleucine (11). The ratio of dehydrodihydroxylysinonorleucine / dehydrohydroxylysinonorleucine is 10:1 in young rats and 4:1 in old rats. With increasing age there is an increase in the reducible cross-links per uterus in rat uterine collagen. Estrogens stimulate the synthesis of these cross-links.

The present study deals with the intermolecular cross-links in the uterine collagens of guinea pig, pig, cow, and human beings.

Materials and methods. Uterine tissues from guinea pig (2 years old), pig (1-5 years), cow (1-4 years), and human beings (25-82 years) were used in this study. Guinea pigs were purchased from Camm Research Institute, Wayne, New Jersey. Pig and cow uteri were obtained from a local slaughter house. The human uterine tissues were samples from surgical hysterectomy or autopsy. With the exception of two leiomyoma samples, all uterine tissues studied were normal. Collagen cross-link standards, dihydroxylysinonorleucine, hydroxylysinonorleucine, lysinonorleucine and histidinohydroxymerodesmosine I and II were generous gifts from Dr. Melvin Tanzer, Connecticut Medical Center, Farmington, Conn., and Dr. A. J. Bailey, Agricultural Research Council, Meat Research Institute, Bristol, England. Tritiated sodium borohydride (320-700 Ci/mole) was purchased from Amersham/Searle Corporation, Des Plaines, Illinois. All chemicals were reagent grade.

Purification of the insoluble collagen, NaB^3H_4 reduction, and separation and identification of the collagen cross-links were as described previously (11). All samples studied were of like size and reduced with like amounts of NaB^3H_4 unless otherwise stated. All preparation procedures were identical for each sample in order to be able to compare the experimental results. Collagen cross-links were identified by elution at the same position with cross-link standards and by cochromatography with the collagen cross-link standards.

Alkaline hydrolysis of the NaB^3H_4 reduced collagen was performed in 2 *N* KOH, 105°, 16 hr. The hydrolysate was neutralized at 5° with 4 *N* $HClO_4$. The reaction mixture was centrifuged, and the supernatant fluid was diluted 10 times with water and desalted on a Dowex 50-X8-H^+ column. Collagen cross-links were eluted with 2 *N* NH_4OH. The eluates were evaporated

[1] VA Projects Nos. 4827-01 and 4827-02.

to dryness under reduced pressure for sample use.

Results. There are three reduced collagen cross-links present in significant amounts in the acid hydrolysates of all the NaB^3H_4 reduced uterine collagen samples studied; namely, dihydroxylysinonorleucine, hydroxylysinonorleucine, and histidinohydroxymerodesmosine (Fig. 1). A small amount of lysinonorleucine is also present in each hydrolysate. In addition, there are three radioactive peaks, A, B, and C, immediately before dihydroxylysinonorleucine, in the position reported for N^{ϵ}-hexosyl-lysine and -hydroxylysine (12, 13).

In the hydrolysate of the NaB^3H_4 reduced uterine collagen of pig and cow (Fig. 1), the dihydroxylysinonorleucine is the principal cross-link. The hydroxylysinonorleucine and the histidinohydroxymerodesmosine are minor cross-links. The peaks A, B, and C are present in small amounts.

In the hydrolysate of the NaB^3H_4 reduced guinea pig uterine collagen, the dihydroxylysinonorleucine is still the major cross-link, even though its radioactivity is relatively lower due to a lower amount of radioactivity used for reduction. The percentages of radioactivities in peaks A, B, and C are relatively higher than those seen in the reduced uterine collagen of pig and cow.

In the hydrolysate of the NaB^3H_4 reduced human uterine collagen, the radioactivities of dihydroxylysinonorleucine, hydroxylysinonorleucine, and histidinohydroxymerodesmosine appear be equal. The radioactivities of peaks A, B, and especially C are greater than those seen in the hydrolysate of reduced uterine collagen of pig, cow and guinea pig.

Table I shows the percentage distribution of the radioactivity in the reduced components of the uterine collagens studied. In the reduced uterine collagen of pig, cow, and guinea pig, approximately 40% of the radioactivity is in dihydroxylysinonorleucine, 20% in hydroxylysinonorleucine, and 15% in histidinohydroxymerodesmosine. The total radioactivity in peaks A, B, and C is about 15–20%. A small percentage of the radioactivity is present in both dihydroxynorleucine and lysinonorleucine. In the hydrolysate of reduced human uterine collagen, the radioactivity in dihydroxylysinonorleucine is approximately 20%, half that of the other species studied. The radioactivity in the hydroxylysinonorleucine is also about 20%. The radioactivity is relatively greater in histidinohydroxymerodesmosine, and in peaks A, B, and C, than in the other species studied. No age differences can be seen in either the percentage distribution of radioactivity in each cross-link or the amount of incorporation of total radioactivity into these cross-links in samples studied between 25–82 years of age.

Table II shows that the radioactivity distribution of the reduced components of collagen from human uterine leiomyoma appears very similar to that from normal human uterine tissue, except that a relatively less percentage of radioactivity is present in component C and a greater percentage of radioactivity is present in dihydroxylysinonorleucine. The incorporation of tritium radioactivity in leiomyoma collagen is greater than that in normal uterine collagen from the same person. The data reveal that leiomyoma collagen is the newly formed collagen produced by uterine fibroblast.

In order to see whether the collagen cross-links occur in uterine tissue as glycosylated compounds as they do in bone, cartilage, and rat skin (14), the tritiated $NaBH_4$ reduced human uterine collagen was hydrolyzed in 2 *N* KOH. The elution pattern of the alkaline hydrolysate is shown in Figs. 2A and 2B. There are two extra radioactive peaks (PA 1 and PA 2) eluted between leucine and phenylalanine in the positions reported for glucosylgalactosyl-dihydroxylysinonorleucine and -hydroxylysinonorleucine (14). There was also an absence of dihydroxylysinonorleucine and a decreased amount of hydroxylysinonorleucine in the base hydrolysate. Peaks PA 1 and PA 2 from several columns were combined, respectively, desalted on a Dowex 50-X8-H^+ column, eluted with 2 *N* NH_4OH, and hydrolyzed in 0.1 *N* HCl and 2 *N* HCl, respectively. The hydrolysates were then rechromatographed. In Fig. 2A, after partial hydrolysis with 0.1 *N* HCl, approximately 60% of PA1 was converted to a compound eluted before NH_3. Upon complete hydrolysis with 2 *N* HCl, approximately 70% of PA 1 was converted to dihydroxylysinonorleucine. Similarly, in Fig. 2B, partial hydrolysis

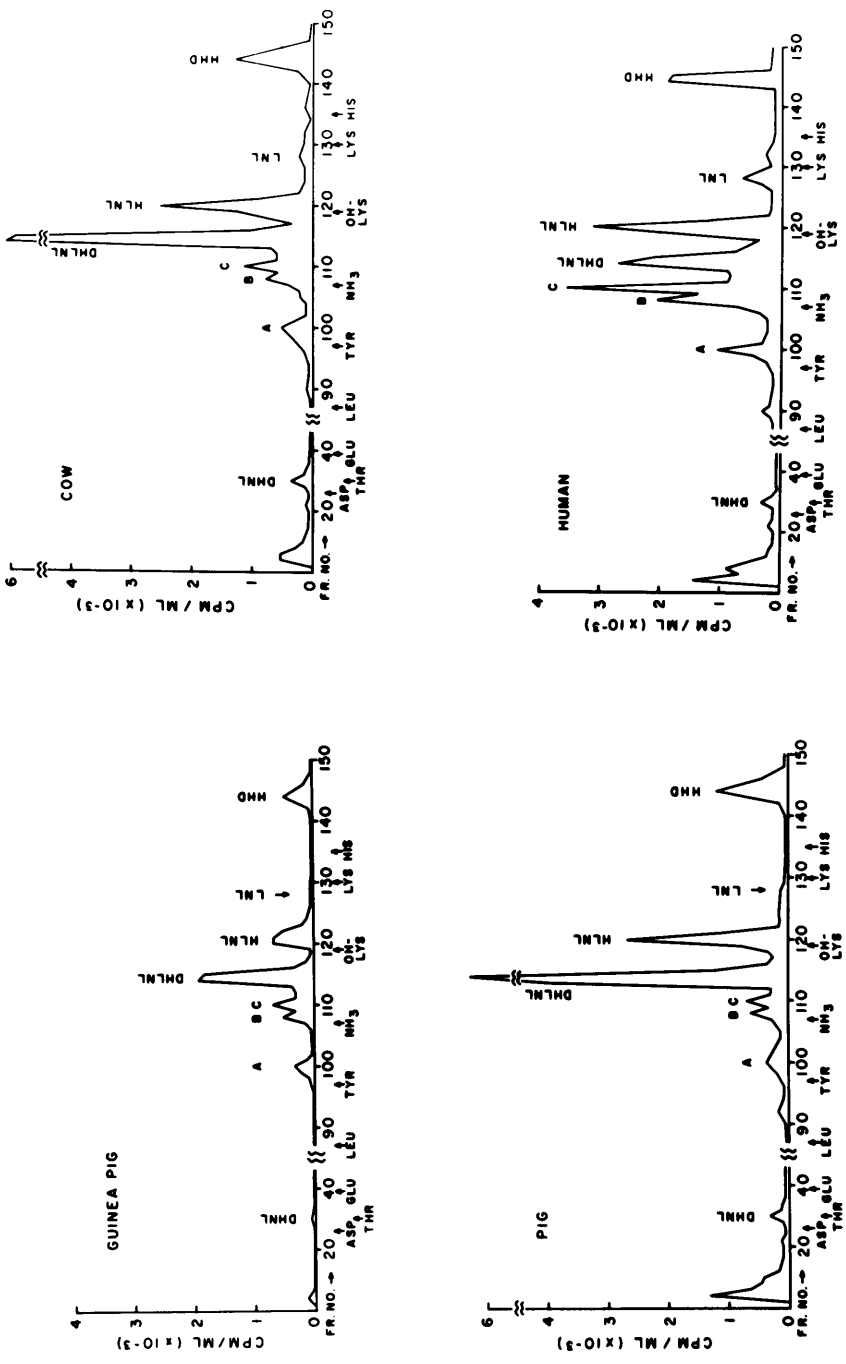

FIG. 1. Elution pattern of acid hydrolysate of NaB^3H_4 reduced uterine collagen. (Fifteen milligrams of purified insoluble uterine collagen was reduced with 1.5 mCi of NaB^3H_4 for pig, cow, and human samples. For the guinea pig sample, 0.75 mCi was used for reduction. Three milligrams of collagen hydrolysate were separated on a split stream Technicon Amino Acid Analyzer. Two milliliter fractions were collected.) Abbreviations: DHNL, dihydroxynorleucine; DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; LNL, lysinonorleucine; HHD, histidinohydroxymerodesmosine.

TABLE I. DISTRIBUTION OF RADIOACTIVITY IN THE REDUCED COMPONENTS OF Uterine COLLAGEN.

Uterine collagen	Number of samples	Percentage							
		DHNL[a]	A	B	C	DHLNL	HLNL	LNL	HHD
Guinea pig	1	3	7	8	9	38	20	3	13
Pig	4	6*	7	6	4	39	20	4	15
		(4-7)**	(6-7)	(5-7)	(4)	(32-47)	(20-21)	(3-5)	(12-16)
Cow	4	5	6	5	6	39	19	4	15
Human		(4-6)	(6-7)	(5-6)	(5-6)	(29-44)	(18-22)	(3-5)	(13-19)
	10	3	9	8	10	20	22	5	24
		(2-4)	(6-11)	(6-12)	(7-12)	(15-27)	(20-26)	(3-7)	(19-30)

[a] Abbreviations: DHNL, dihydroxynorleucine; DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; LNL, lysinonorleucine; HHD, histidinohydroxymerodesmosine.

* Mean.

** Range.

TABLE II. RADIOACTIVITY DISTRIBUTION OF THE REDUCED COMPONENTS OF COLLAGEN IN NORMAL AND LEIOMYOMA UTERINE TISSUE.

Uterine collagen	Percentage								Total cpm samples counted
	DHNL[a]	A	B	C	DHLNL	HLNL	LNL	HHD	
Case 1									
Normal	4	11	10	12	18	19	7	19	28,700
Leiomyoma	2	11	4	7	20	21	7	28	40,000
Case 2									
Normal	2	8	5	13	16	22	5	29	37,300
Leiomyoma	2	7	4	8	23	23	4	29	42,900

[a] Abbreviations: DHNL, dihydroxynorleucine; DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; LNL, lysinonorleucine; HHD, histidinohydroxymerodesmosine.

of PA 2 resulted in producing a compound eluted before NH_3. Complete hydrolysis of PA 2 resulted in the formation of hydroxylysinonorleucine (70%). The elution position of PA 1 and PA 2 appear to be at the reported position for glucosygalactosyl-dihydroxylysinonorleucine and -hydroxylysinonorleucine and the elution positions of the peaks before NH_3 are at the positions reported for galactosyl-dihydroxylysinonorleucine and -hydroxylysinonorleucine (14). These data suggest that the dihydroxylysinonorleucine and hydroxylysinonorleucine are present *in vivo* as glucosyl-galactosyl complexes, as reported by Robins and Bailey (14) in bone, cartilage, and skin, and in sea cucumber body wall collagen as reported by Eyre and Glimcher (15). PA 1 and PA 2 were also present in the alkaline hydrolysate of tritiated $NaBH_4$ reduced uterine collagen of cow and rat.

Discussion. Previous work (11) on rat uterine collagen revealed that there are only two reducible cross-links, dehydrodihydroxylysinonorleucine and dehydrohydroxylysinonorleucine. In the present study an additional reducible cross-link, histidinohydroxymerodesmosine appears in all the uterine collagens studied. The percentage of histidinohydroxymerodesmosine is highest in the reduced human uterine collagen. Dehydrodihydroxylysinonorleucine is the principal cross-link in the uterine collagens of guinea pig, pig, and cow. Only in human uterine collagen is dihydroxylysinonorleucine present in equal amounts as hydroxylysinonorleucine and histidinohydroxymerodesmosine. These differences are attributed either to the species or age of the animals studied.

Alkaline hydrolysis reveals that both dehydrodihydroxylysinonorleucine and dehydrohydroxylysinonorleucine occur *in vivo* as glycosylated complexes, which may be converted to partially hydrolyzed galactosyl cross-links with 0.1 *N* HCl and to the free cross-links upon 2 *N* HCl hydrolysis. The elution positions of PA 1 and PA 2 coincide with those reported for glucosylgalactosyl-dihydroxylysinonorleucine and -hydroxylysinonorleucine, and the peaks eluted before NH_3 coincide with galactosyl-dihydroxylysinonorleucine and -hydroxylsinonorleucine (14). Therefore, similar to the dehydrodihy-

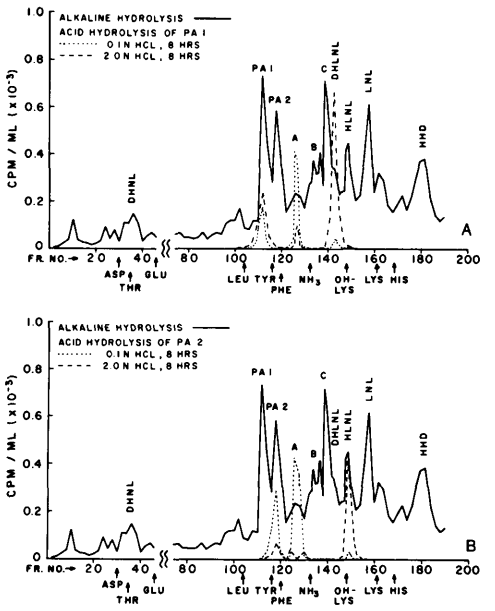

FIG. 2. Elution pattern of alkaline hydrolysate of NaB^3H_4 reduced human uterine collagen. (Three milligrams collagen hydrolysate were separated on a split stream Technicon Amino Acid Analyzer; 1.5-ml fractions were collected.) Abbreviations: same as in Fig. 1. Fig. 2A, Acid hydrolysis of PA 1; Fig. 2B, acid hydrolysis of PA 2.

droxylysinonorleucine in bone and cartilage and dehydrohydroxylysinonorleucine in skin, both of these cross-links in uterine collagen are present as glycosylated complexes *in vivo*.

In the amino acid chromatograms there were two ninhydrin positive peaks seen in the position of histidinohydroxymerodesmosine in the acid hydrolysate of each uterine collagen samples studied. Since the *in vivo* reduction of dehydrodihydroxylysinonorleucine was reported in bone and cartilage (4), in order to determine whether there was any *in vivo* reduction in uterine collagen, 15 mg of human uterine collagen was hydrolyzed without reduction and chromatographed. No ninhydrin positive peaks were found. Therefore, the *in vivo* reduction of this collagen crosslink does not occur in human uterine collagen.

The present study reveals species differences in the type of uterine collagen crosslink present. Even at 82 years of age, there are large amounts of reducible cross-links in human uterine collagen.

Summary. The intermolecular cross-links have been studied in the uterine insoluble collagen of guinea pig, pig, cow, and human beings with a single given procedure. After NaB^3H_4 reduction, there are three intermolecular cross-links; namely, dihydroxylysinonorleucine, hydroxylysinonorleucine, and histidinohydroxymerodesmosine. In human uterine collagen samples these reduced cross-links are present in equal amounts. The reduced intermolecular collagen cross-links of uterine leiomyoma are very similar to those of the normal uterine tissue. Dihydroxylysinonorleucine is the principal reduced cross-link in uterine collagen of guinea pig, pig, and cow. Alkaline hydrolysis reveals that dehydrodihydroxylysinonorleucine and dehydrohydroxylysinonorleucine occur *in vivo* as glycosylated derivatives.

The authors gratefully acknowledge the supplying of sample materials by Dr. Amy Huang, Fairfax Hospital, Falls Church, Virginia, and Dr. Chien Wei Lan, St. Elizabeths Hospital, Washington, D.C.

1. Bailey, A. J., and Peach, C. M., Biochem. Biophys. Res. Commun. **33,** 812 (1968).
2. Bailey, A. J., Fowler, L. J., and Peach, C. M., Biochem. Biophys. Res. Commun. **35,** 663 (1969).
3. Tanzer, M. L., and Mechanic, G., Biochem. Biophys. Res. Commun. **39,** 183 (1970).
4. Mechanic, G., Gallop, P. M., and Tanzer, M. L., Biochem. Biophys. Res. Commun. **45,** 644 (1971).
5. Fairweather, R. B., Tanzer, M. L., and Gallop, P. M., Biochem. Biophys. Res. Commun. **48,** 1311 (1972).
6. Tanzer, M. L., Housley, T., Berube, L., Fairweather, R., Franzblau, C., and Gallop, P. M., J. Biol. Chem. **248,** 393 (1973).
7. Tanzer, M. L., Fairweather, R., and Gallop, P. M., Biochim. Biophys. Acta **310,** 130 (1973).
8. Kao, K. Y. T., Hilker, D. M., and McGavack, T. H.,Proc. Soc. Exp. Biol. Med. **106,** 121 (1961).
9. Woessner, J. F., Jr., Biochem. J. **83,** 304 (1962).
10. Woessner, J. F., Jr., and Brewer, T. H., Biochem. J. **89,** 75 (1963).
11. Kao, K. Y. T., and Hitt, W. E., Biochim. Biophys. Acta **371,** 501 (1974).
12. Tanzer, M. L., Fairweather, R., and Gallop, P. M., Arch. Biochem. Biophys. **151,** 137 (1972).
13. Robins, S. P., and Bailey, A. J., Biochem. Biophys. Res. Commun. **48,** 67 (1972).
14. Robins, S. P., and Bailey, A. J., FEBS Lett. **38,** 334 (1974).
15. Eyre, D. R., and Glimcher, M. J., Proc. Soc. Exp. Biol. Med. **144,** 400 (1973).

Received July 25, 1975. P.S.E.B.M. 1976, Vol. 151.