Rate of Collagen Formation in Biopsy-Connective Tissue of the Rat. (23279)

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The rate of collagen formation has been difficult to determine in man and animal. Recently, collagen formation has been studied in granulomatous lesions induced by the injection of an irritant, such as carrageenin(1). When the sponge implantation technic(2) is used for isolation of connective tissue, the age of the newly developed connective tissue is known and rate of collagen formation may be quantitated. Tissue response to the plastic sponge implant has been followed in the albino rat histologically and biochemically, and the accumulated data indicate a predictable and reproducible reaction, which results in formation of new normal-appearing connective tissue throughout the interstices of the sponge. In this paper, rate of collagen formation and degree of acetic acid solubilization of collagen in 7 to 300-day-old connective tissue obtained from male and female albino rats will be presented.

The biological, biochemical and physical chemical characteristics of connective tissue are becoming of increasing interest in medical research because changes which occur with age have been noted in connective tissue(3). Furthermore, changes in connective tissue of the wall of the artery appear to precede the lesion of atherosclerosis(4).

Methods. Adult male and female albino rats were implanted with 3 sponges each along the dorsum(2). The biopsy-connective tissues from 3 male and 3 female rats were removed at 7, 14 and 21 days after implantation and from another group of 3 male rats at 10, 17 and 24 days. The connective tissue biopsies were removed from 5 male and 5 female rats after 100 days and from a male and a female rat after 300 days of tissue growth. After removal of the sponge implants, the tissue biopsy was frozen at –26°C in order to facilitate the removal of the capsule. The sponge-connective tissue was weighed and homogenized in physiological saline (pH 7.4) in a VirTis homogenizer with a rheostat setting of 50 for 5 minutes. The resulting homogenate was centrifuged at 13,000 rpm at 5°C for one hour to separate the saline-soluble fraction of the tissue from the residue. After decanting the supernatant, the residue was washed once with distilled water at 5°C and centrifuged at 13,000 rpm at 5°C for half an hour. The water wash was discarded. The saline-insoluble residue was hydrolyzed with 6 N HCl in a sealed pyrex tube for 16 hours at 110°C. The nitrogen concentrations of the saline homogenate and the acid hydrolysate of the residue were determined by the Conway microdiffusion technic(5) and converted to protein by the usual factor of 6.25. Hydroxyproline, which was determined by the method of Neuman and Logan(6), was multiplied by the factor of 7.46 to obtain the collagen value. The non-collagenous protein of the residue was obtained by subtracting the collagen value from the total protein value for the residue. One-half molar acetic acid was used for the solubilization of the saline-insoluble protein. The insoluble protein was extracted at 5°C for 3 successive 24-hour periods. After each extraction, the sample was centrifuged at 29,500 rpm at 5°C for 1 hour in a Spinco Model L Ultracentrifuge to obtain the acetic acid-soluble fraction. Protein contents of the acetic acid-soluble and -insoluble fractions were determined. All data were calculated on the basis of weight of the dry sponge implanted.

Results. The content of protein and percentage distribution of the protein of the rat sponge-connective tissue at 7 to 300 days of tissue age are recorded as mean values in Table I. The collagenous protein of the saline-insoluble residue increased rapidly during the first 21 days of growth in both sexes. Beyond 21 days of tissue age, the collagen of the connective tissue from the male rat continued to increase while in the female rat, the collagen content remained relatively constant.

When the collagen content of the newly

TABLE I. Protein of Rat Biopsy-Connective Tissue.

	Tissue age (days)	mg/g sponge				% distribution		
			Saline-insoluble				Saline-insoluble	
		Saline-soluble	Collagen	Non-collagen	Total	Saline-soluble	Collagen	Non-collagen
♂	7	335	11*	173	519	64	2	34
	10	418	37	200	655	63	6	31
	14	288	77	187	552	56	14	30
	17	382	113	224	719	53	15	32
	21	321	180	198	699	46	26	28
	100	349	386*	195	930	37	42	21
	300	666	475	314	1455	46	33	21
♀	7	409	28*	176	613	65	4	31
	14	384	103	218	705	57	15	28
	21	349	169	201	719	49	23	28
	100	353	198*	248	799	46	28	26
	300	400	160	193	753	53	21	26

* $p < 0.05$ when male and female data compared.

forming connective tissue was plotted against the age of the tissue in days on semilogarithmic scale (Fig. 1), there was a linear relationship between collagen content and tissue age during the initial 14-day period in the female tissue and during the 21-day period in the tissue of the male. The slopes and rate constants of the linear portions of the 2 curves were the same.

The effect of acetic acid upon the saline-insoluble protein of the biopsy-connective tissue as the tissue ages is shown in Table II. The acetic acid-solubilized protein increased in amount from 7 to 300 days of tissue age. The increase in the tissue of the female was not as great as in the tissue of the male. However, the percentage of the total saline-insoluble protein which was solubilized by acetic acid decreased with connective tissue age in both sexes, and this reduction in solubilization at 300 days' tissue growth was more marked in the male than in the female (Table II).

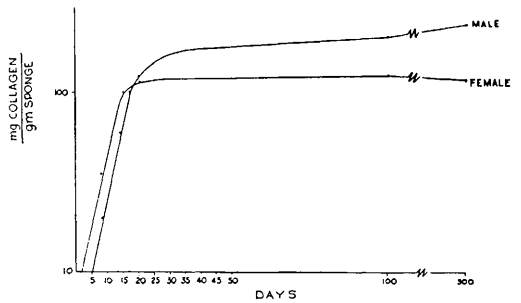

FIG. 1. Rate of development of collagen of rat biopsy-connective tissue.

Discussion. Following implantation of the sponge into the rat, a wall of fibroblasts and amorphous material envelops it as a capsule and the interior of the sponge becomes filled with an eosin-staining material. At the 4th to 5th day following sponge implantation, the eosin-staining material coalesces into strands, which are argentophilic. The fibroblasts then move into the sponge and occupy the periphery of the sponge by the 7th day. At the 7th day, hydroxyproline was detected in the connective tissue biopsy which had been freed of its capsule. The presence of this amino acid is indicative of the beginning of the collagen structure.

Work reported from these laboratories indicated an electrophoretic similarity between saline-soluble protein of the tissue and serum protein(7). This soluble fraction of the tissue contained protein components which migrated to the albumin, the alpha, beta and gamma globulin zones of serum run simultaneously. The protein of the saline-soluble fraction is apparently derived from the serum within the first few days following sponge implantation and remains in equilibrium with the serum during the 100 to 300 days of connective tissue development.

The non-collagenous protein does not appear to be an essential part of the collagen structure because it is separated from the collagen of the acetic acid-soluble fraction of the connective tissue as the latter precipitates

TABLE II. Saline-Insoluble Protein of Rat Biopsy-Connective Tissue.

Tissue age (days)	Total, mg/g sponge ♂	Total, mg/g sponge ♀	Acetic acid-soluble, mg/g sponge ♂	Acetic acid-soluble, mg/g sponge ♀	% solubilized ♂	% solubilized ♀
7	142.2 ± 15.6 (3)*	164.6 ± 44.0 (3)	35.7 ± 7.1	52.0 ± 15.1	24.9 ± 3.2	31.8 ± 4.1
21	398.4 ± 53.1 (6)	373.6 ± 38.2 (9)	63.5 ± 17.3	38.0 ± 8.0	16.2 ± 5.5	10.3 ± 2.0
300	1455.2 (1)	369.4 (1)	153.0	70.0	12.6	19.7

* Figure in parenthesis indicates No. of tissues.

during dialysis. The crystalline-like strands of collagen which then form have the characteristic hydroxyproline concentration of 13.6% and an intrinsic viscosity of 12 to 13. Yet the non-collagenous protein of the acetic acid-soluble fraction is apparently firmly bound to collagen because the acid-soluble material behaves as a monomer during ultracentrifugation at 250,000 G for one hour at 6°C.

Factors which control rate of collagen synthesis are not understood. The fact that the collagen content of the tissue from the female remained constant after 21 days of growth would appear to indicate a slow rate of collagen formation as suggested by studies on incorporation of C^{14}-labeled glycine in tendons (8). It might also indicate that collagen synthesis rate in the connective tissue of the female equals rate of collagen breakdown. Current research in our laboratories indicates that it is this latter state of equilibrium which probably occurs. The connective tissue from the male continued to increase in collagen content up to 300 days of tissue growth. Apparently in the male rat, rate of collagen synthesis surpasses rate of collagen degradation. Some factor, presumably hormonal, apparently enhances the synthesis and breakdown of collagen in connective tissue from the female rat, and it would appear that this factor is less active in the male rat.

The percentage of scleroprotein solubilized by acetic acid decreased with tissue age, and this finding is similar to that reported by Orekhovich(3) and Nageotte(9) in their studies on the acid extraction of skin. This tissue age difference in the solubility of collagen is thought to be related to the degree of cross-linking of the polypeptide strands. It is also noted that the 300-day-old tissue from the female rat had a lower collagen content and yet a greater percentage of solubilized collagen than did the tissue of the male. This suggests that not only is the collagen of the female rat metabolically more active than the collagen of the male but that the internal bonding of the collagen obtained from the female rat is more easily disrupted by weak acids than that of the male.

Summary. (1) Collagen was found initially in connective tissue biopsy of male and female rats at 7 days following sponge implantation. (2) Collagen content of connective tissue biopsy increased rapidly in both sexes during the first 14 days of tissue growth. (3) After 21 days of connective tissue development in the female, collagen content remained constant up to 300 days of tissue age while in the male, collagen content continued to increase as the tissue aged. (4) The percentage of total scleroprotein which was solubilized by 0.5 M acetic acid decreased with tissue age and this decrease was greater in the 300-day-old tissue of the male rat than in comparable tissue of the female.

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Effect of Alternating Periods of Underfeeding and *Ad libitum* Feeding on Insulin Sensitivity of Rats.* (23280)

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Prolonged underfeeding (4-6 weeks on 10 g of diet daily) followed by a 24 hr fast causes an increase in the hypoglycemic response to insulin in adult male rats(1). In the present study fasting insulin sensitivity of a group of rats was examined after a period of undernutrition, during a subsequently instituted *ad libitum* feeding regimen, and in the course of a second period of undernutrition.

Materials and methods. Seven male Sprague-Dawley rats, serving as their own controls, were used. The mean initial weight of the group was 281 ± 4 (S.E.) g. All insulin sensitivity determinations were undertaken after a 24 hr fast. One tenth U of insulin (Iletin-Lilly) per kg was injected i.p. Blood sugar levels were ascertained as in our previous study(1). The first insulin sensitivity measurement (Fig. 1, A) was performed 5 weeks after the initiation of a restricted feeding regimen (10 g ground Rockland diet daily), the second (Fig. 1, B) after 3 weeks and the third (Fig. 1, C) after 6 weeks of subsequent *ad libitum* feeding of the same diet. Following the third determination daily food rations were again reduced by 2 g daily from

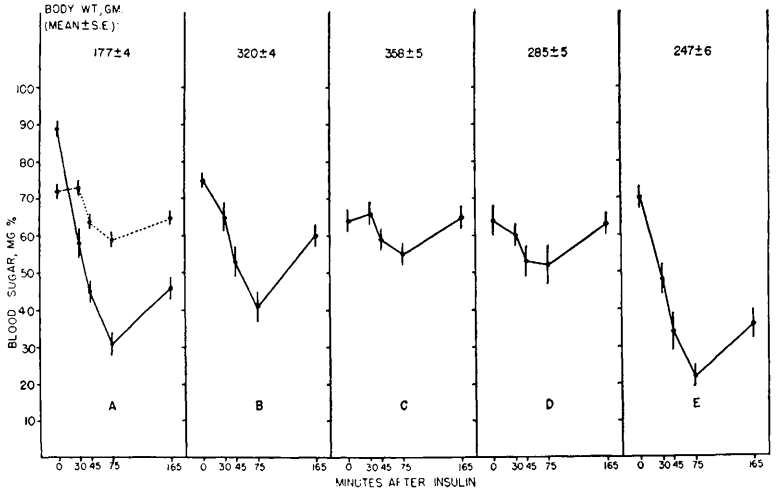

FIG. 1. Blood sugar levels before and after insulin in fasted rats after prolonged undernutrition (A), during *ad libitum* feeding (B and C) and renewed undernutrition (D and E). Details in text. Dotted line: values in 23 *ad libitum* fed-fasted rats of a previous study(1). Vertical lines: stand. errors of the mean.

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