Determination of the Number of Crosslinks in Collagens from Mechanical Properties of Swollen Fibers

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SYNOPSIS

A method for the determination of crosslink density for collagen fibers was proposed. The number of interchain crosslinkages in whale ligament and rat-tail tendon was estimated by applying an usual rubber elasticity theory. Collagen fibers swollen in a solution composed of equal volumes of 8*M* LiBr aqueous solution and diethylene glycol monoalkyl ether showed a typical rubber elasticity. The energy components to total retractive forces were similar in order in magnitude for crosslinked natural rubbers, namely, below 0.3. It was found that no intermolecular crosslinkage occurs between tropocollagen molecules in tendon from a 2-month-old rat, while there are about 12 crosslinking sites per molecule in tendon from a 10-month-old rat and 15 sites in whale ligament. The number, type, and crosslinking sites in the tendon crosslinked with 1,3-bis(vinylsulfonyl)-2-propanol is also discussed. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

Collagen is one of the most abundant proteins in nature. Collagen is the principal fibrous component of cartilage, ligaments, and tendons. The collagen molecule consists of three helical polypeptide chains (α chains) interwound with one another in such a way as to form a rodlike superhelix structure. These rods are termed tropocollagen. Five tropocolagen units are assembled in a quarter stagger to form a microfibril[1,2] and the collagen fibril consists of microfibrils packed in a tetragonal lattice.[3,4] It is known that covalent intramolecular and intermolecular crosslinks are formed in the collagen fibril.[5-8] The former occur between the chains of the same tropocollagen molecule, and the latter, between adjacent tropocollagen molecules. The collagen molecules are then stabilized in the fibrils by these covalent crosslinkages and these are vital for normal physiological function. Mechanical properties such as tensile strength and viscoelasticity of the connective tissue matrices are related directly to the amount of intermolecular crosslinkages.[7]

Many types of intermolecular or interchain aldol crosslinks resulted from an enzyme-mediated reaction and Schiff base crosslinks originated from juxtaposed specific peptidyl residues of lysine, hydroxyl lysine, and histidine have been identified in collagen.[9,10] Fujimoto et al.[11-13] found new crosslinks of pyridinoline that links three chains in collagen molecules[14] and is predominant in skeletal tissues such as tendon and ligament.

Cater[15] reported a mechanical method for evaluating the crosslink density of collagen crosslinked with aldehydes or other difunctional agents by measuring the modulus of elasticity in water for tanned and untanned collagens denatured in boiling water. The crosslink density obtained is, however, only relative since the denatured collagen fibers are viscoelastic in water.

One of authors established a method for the determination of the crosslink density by measuring the modulus of elasticity of the protein fibers in a mixed solution composed of concentrated aqueous LiBr solution and diethylene glycol monoalkyl ether.[16] By means of mechanical and chemical methods, the number, type, and location of crosslinks in keratin fibers[17-19] and artificially crosslinked silk fibroin fibers[20] were demonstrated. The aim of

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this study was to investigate a swelling medium showing an ideal or nearly ideal rubber elasticity for collagen fibers, to determine the crosslink density of various collagens by applying an usual rubber elasticity theory, and to compare the number of naturally occurring crosslinkages among collagens. The number, type, and sites of crosslinkages in artificially crosslinked collagen fibers will be discussed.

EXPERIMENTAL

Materials

Whale ligament stored in dry state for about 30 years was kindly supplied by the Technical Center for Leather, Hyogo Prefectural Institute of Industrial. The materials were Soxhlet-extracted with acetone for about 24 h and followed by washing with water and air-drying. Test samples were prepared from a thick and flat ligament to separate into a fine fibrous form. Collagens from tail tendons of Wister rats of ages 2 months (RTT2) and 10 months (RTT10) were used. The tail tendons were separated from the rat tail under little tension and immediately immersed into a solution composed of equal volumes of *n*-propanol and water and then stored in the same solution at 5°C.

1,3-Bis(vinylsulfonyl)-2-propanol (BVSP) used as a crosslinking agent was prepared according to the method of Sera et al.[21] The compound was purified by recrystallization from ethanol twice before use; mp: 98–99°C.

ANAL: Calcd for $C_7H_{12}O_5S_2$: C, 34.99%; H, 5.03%. Found: C, 35.02%; H, 4.97%. ^{1}H-NMR(acetone-d_6): 3.44 (m, 4H, $-CH_2-SO_2$), 4.69 (m, 1H, $=CH-O$), 4.85 (d, 1H, OH), 6.28 (dd, 4H, $CH_2=$), 6.94 (m, 2H, $=CH-$). ^{13}C-NMR (acetone-d_6): 60.17($-CH_2-$), 63.70(CH—OH), 129.55($CH_2=$), 139.147($=CH-$).

Diethylene glycol monobutyl ether (BC) and diethylene glycol nonomethyl ether (MC) were obtained by distillation of commercial products under reduced pressure after dehydration with anhydrous sodium sulfate as previously reported.[17]

Preparation of RTT Collagen Crosslinked with BVSP

After excess liquid was removed from the RTT2 collagen stored in a solution composed of equal volumes of *n*-propanol and water, about 0.1 g of the tendon was immersed into 20 mL of a 0.1*M* BVSP solution composed of equal volumes of *n*-propanol and phosphate buffer solution adjusted at pH 8.0 under gentle stirring for 24 h at 35°C and followed by washing with water.

Density Measurements of Collagen

The density of collagen was measured by using a density gradient column with ethanol–carbon tetrachloride at 25°C.

Amino Acid Analysis

The BVSP-treated and untreated collagen fibers (5 mg each) were hydrolyzed with 6*M* HCl (2 mL) for 24 h at 110°C in deaerated conditions, dried under reduced pressure, and then diluted with 0.02*M* HCl (2 mL) for amino acid analysis. The amino acid analyzer used was a Hitachi automatic high-speed amino acid analyzer type L-8500.

Preparation of Swollen Collagen

The collagen samples were swollen at room temperature in a solution composed of equal volumes of 8*M* LiBr aqueous solution and BC or MC. The swollen samples were subjected to mechanical tests. The cross-sectional area of swollen collagen sample, S, was determined by eq. (1):

$$S = (W_0/L_s\rho)(v_2^{-1} - 1) \quad (1)$$

where W_0 is the weight of the dry sample; L_s, the swollen length of the sample; ρ, the density of collagen sample; and v_2, the volume fraction of collagen in swollen sample. The v_2 value was estimated by assuming the additivity of specific volume of collagen and dilutent in the fiber as in eq. (2):

$$v_2 = W_0\rho_d/[W\rho - W_0(\rho - \rho_d)] \quad (2)$$

where W is the weight of swollen sample in equilibrium at 25°C, and ρ_d, the density of the diluent. The values of ρ_d at 25°C were 1.243 and 1.286 g/cm^3 for 8*M* LiBr–BC and LiBr–MC solutions, respectively.

Mechanical Tests for Swollen Collagen

The mechanical tests for the swollen samples were carried out by using a Model UTM-II Tensilon tester (Toyo Baldwin Co.) equipped with an apparatus as shown in Figure 1. Each of the swollen samples with about 20 mm length was set between clamps. The upper clamp was attached to a load cell. The lower clamp could be moved downward so as to impose a

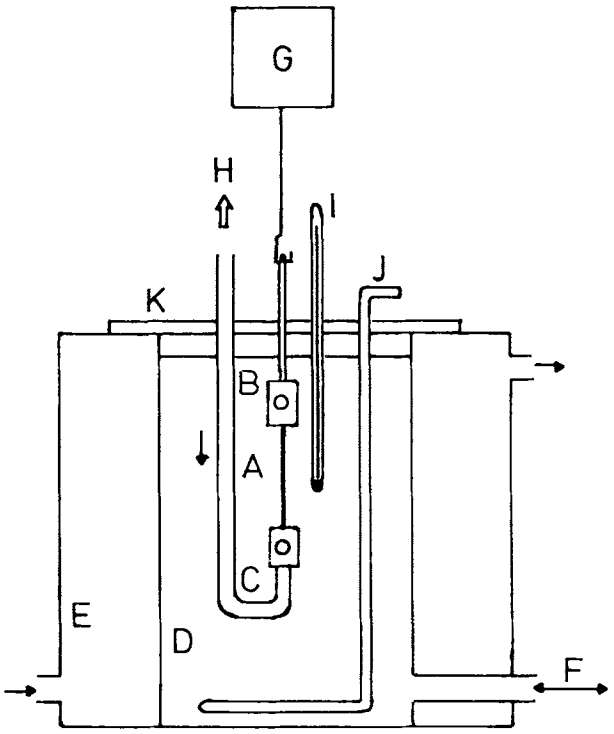


Figure 1 Apparatus used for mechanical measurements of swollen fiber samples: (A) swollen sample; (B,C) clamps (quartz); (D) glass cell; (E) water jacket; (F) level regulation; (G) load cell; (H) crosshead; (I) thermometer; (J) stirrer; (K) glass cover.

certain elongation. The sample was placed in a double-jacket cell containing the test solution. The temperature was controlled by circulating the water in the jacket at constant temperature within ±0.1°C.

Stress–Strain Measurements

The stress–strain relations for the swollen samples of about 20 mm in length were obtained at a constant temperature and at a constant extension speed of 2 mm/min. Stresses referred to the cross-sectional area of the swollen and unstrained fiber were used to construct the stress–strain curve.

Stress–Temperature Relationship

The sample was elongated at an extension speed of 2 mm/min to the maximum length and conditioned by repeated loading and unloading at the highest temperature used. To obtain the force–temperature relationships, equilibrium forces were measured at constant temperatures and at constant elongations. First, the unstrained zero length at equilibrium L_0 of the sample being conditioned at the maximum temperature was determined by extrapolating the forces under very small elongations to zero force. Strain ratios were calculated by the equation $\lambda = 1 + L/L_0$. Next, the sample was elongated to length L_1, which corresponds to the strain ratio λ_1 at this temperature and allowed to relax for t_1 until an equilibrium force f_1 in grams was obtained. The sample was further elongated to L_2, (λ_2), L_3, (λ_3), . . . , and equilibrium forces $f_2, f_3, \ldots$, were obtained after relaxing for $t_2, t_3, \ldots$, respectively. Relaxation times required for measurement of equilibrium forces were more than 1 min, but different among the strain ratios. The longest relaxation time observed at maximum extension of the swollen fiber was about 5 min. The temperature was then lowered at intervals of about 5°C and the corresponding equilibrium forces were measured at constant strain ratios and at constant temperatures as described above. Finally, the temperature was increased again at about 10°C intervals.

RESULTS

Stress–Strain Curves

Figure 2 shows the stress–strain curves of collagen samples conditioned by three cycles of loading and unloading. For any swollen samples of collagen, ex-

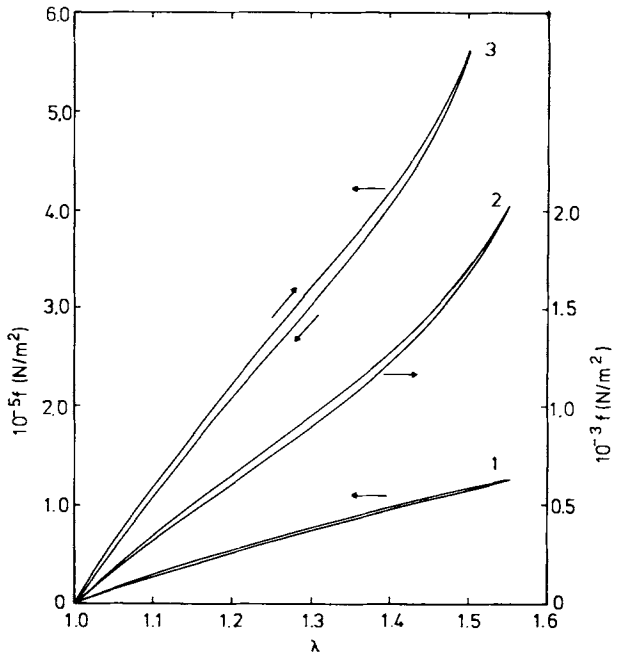


Figure 2 Hysteresis curves of swollen collagen fibers in 8*M* LiBr–BC solution: (1) whale ligament; (2) rat-tail tendon (RTT2); (3) RTT2 crosslinked with BVSP.

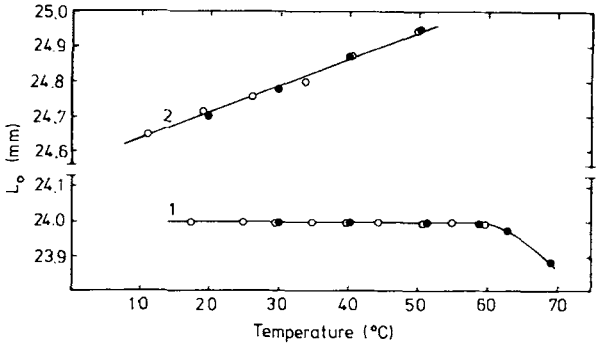


Figure 3 Unstrained swollen length at equilibrium, L_0, vs. temperature curve: (1) whale ligament in 8*M* LiBr–BC solution; (2) BVSP crosslinked rat-tail tendon (RTT2) in 8*M* LiBr–MC solution.

cellent elastic recoveries and lower energy losses are observed. These properties are characteristic of rubber materials.

Thermal Expansion Coefficient

Figure 3 shows the change of the unstrained zero length (L_0) with temperature. As seen in Figure 3, no change of L_0 is observed for the whale ligament collagen in 8*M* LiBr/BC over the range of temperature from 20 to 60°C. Therefore, it could be assumed that the bulk coefficient of thermal expansion, $\beta_{eq} = V_0^{-1}(\partial V/\partial T)_{p,eq}$, of the swollen collagen is substantially zero, where V_0 is the unstrained, swollen volume of the sample at equilibrium. Deviations of the plots from the straight line at higher temperatures than 60°C occur. It has been reported that in the 8*M* LiBr–BC diluent system the value of β_{eq} is zero for hair and chemically modified keratins.[17] Similar results were also obtained for RTT collagen crosslinked with BVSP.

On the other hand, the length L_0 of the crosslinked RTT2 collagen is linearly increased with increasing temperature in the 8*M* LiBr–MC system. The linear coefficient of thermal expansion, $\beta_{eq}/3 = L_0^{-1}(\partial L/\partial T)_{p,eq}$, is calculated to be 3.24×10^{-4} deg^{-1}.

Force–Temperature Relationship

Figures 4 and 5 show straight force–temperature curves for whale ligament in 8*M* LiBr–BC and crosslinked RTT collagen in the 8*M* LiBr–MC solution, respectively. The data taken at decreasing and increasing temperature coincide on the straight lines at constant extensions.

DISCUSSION

Force–Temperature Relationship of Collagen

The force–temperature coefficient at a constant pressure and a constant strain ratio in equilibrium corresponding to that at constant volume and constant length is expressed for the Gaussian network by eq. (3)[22,23]:

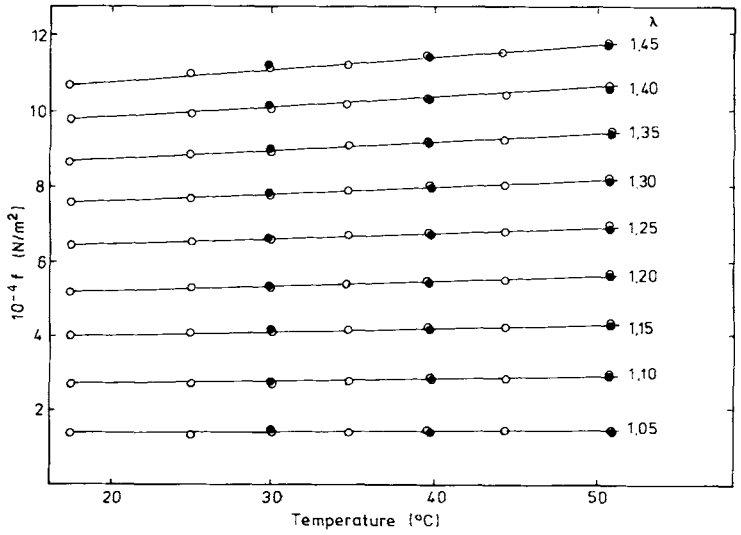


Figure 4 Equilibrium force, *f*, vs. temperature curves at different extension ratios, λ, for whale ligament in 8*M* LiBr–BC solution: (○) temperature descending; (●) temperature ascending.

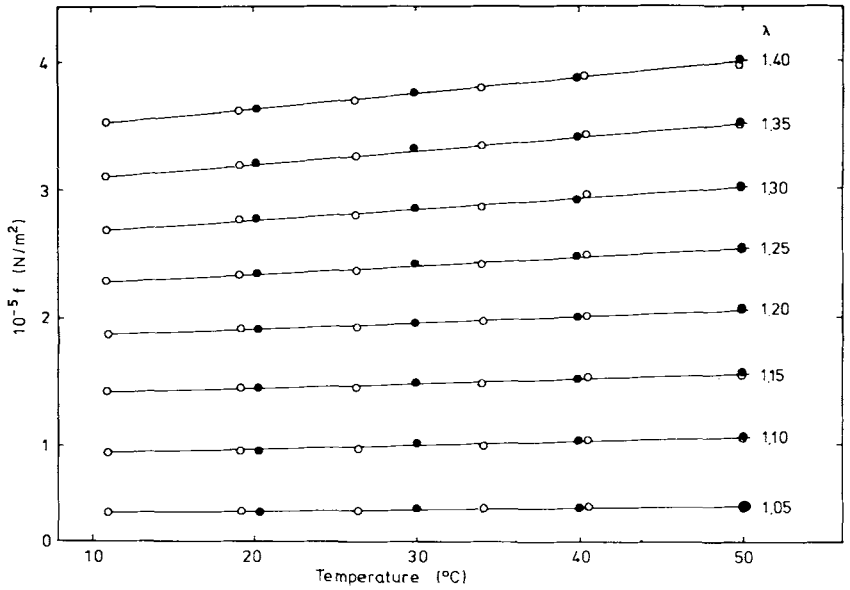


Figure 5 Equilibrium force, f, vs. temperature curves at different extension ratios, λ, for BVSP crosslinked rat-tail tendon (RTT2) in 8*M* LiBr–MC solution: (○) temperature descending; (●) temperature ascending.

$$(\partial f/\partial T)_{p,\alpha,eq} = (\partial f/\partial T)_{V,L,N} + f\beta_{eq}/3 \quad (3)$$

where f is the total force; $\alpha = L/L_i$, the strain ratio with respect to the undistorted state of volume V, representing the volume in the final stressed state; L, the length in the strained state, and L_i, the length in the undistorted state at the volume V; N, the mol number of the diluent in the polymer network; and β_{eq}, the bulk coefficient of thermal expansion at no extension in equilibrium.

Since the force–temperature coefficient actually measured, $(\partial f/\partial T)_{p,\lambda,eq}$, will differ from $(\partial f/\partial T)_{p,\alpha,eq}$, but the difference is only to a negligible extent,[24] the former value can be taken as nearly equal to the right-hand side of eq. (3). The ratio of the energy component, $f_e \equiv (\partial E/\partial L)_{V,T,N}$, to the total equilibrium force f, can be therefore expressed by eq. (4):

$$f_e/f \cong 1 - (T/f)(\partial f/\partial T)_{p,\lambda,eq} + T\beta_{eq}/3 \quad (4)$$

At $\beta_{eq} = 0$, f_e/f is expressed simply by eq. (5):

$$f_e/f \cong 1 - (T/f)(\partial f/\partial T)_{p,\lambda,eq} \quad (5)$$

Table I shows the results obtained for whale ligament in 8*M* LiBr–BC. The quantity f_e/f calculated by eq. (5) is almost independent of extension ratios

Table I Thermoelastic Data for Whale Ligament in Equilibrium Swelling with 8*M* LiBr–BC Solution in the Temperature Range 20–50°C

$\lambda_{40°C}$	$10^{-4} f$ (N/m²)	10^{-1} $(\partial f/\partial T)_{p,\lambda,eq}$	$(T/f)(\partial f/\partial T)_{p,\lambda,eq}$	f_e/f
1.05	1.35	2.51	0.582	0.42
1.10	2.70	7.51	0.871	0.13
1.15	4.00	9.93	0.777	0.22
1.20	5.16	12.2	0.739	0.26
1.25	6.36	13.9	0.685	0.32
1.30	7.56	16.6	0.689	0.31
1.35	8.68	19.2	0.692	0.31
1.40	9.80	22.0	0.702	0.30
1.45	10.85	26.6	0.766	0.23
				Av. 0.28

Table II Thermoelastic Data for Rat-tail Tendon Crosslinked with BVSP in Equilibrium Swelling with 8*M* LiBr–MC Solution in the Temperature Range 10–50°C

$\lambda_{40°C}$	$10^{-4} f$ (N/m^2)	$10^{-2} (\partial f/\partial T)_{p,\lambda,eq}$	$(T/f)(\partial f/\partial T)_{p,\lambda,eq}$	f_e/f
1.05	5.10	1.32	0.808	0.29
1.10	10.3	2.78	0.846	0.26
1.15	15.2	3.57	0.737	0.36
1.20	20.0	4.88	0.764	0.34
1.25	24.7	6.67	0.847	0.25
1.30	29.3	8.47	0.906	0.20
1.35	34.0	10.3	0.951	0.15
1.40	38.9	12.2	0.983	0.12
				Av. 0.25

and the average value is 0.28, which is similar to the values reported for crosslinked natural rubbers.[24]

Table II shows the results obtained for RTT2 collagen crosslinked with BVSP. The average value for f_e/f estimated by applying eq. (4) is 0.25. It is notable that the retractive force arising from the swollen network is nearly entropic even at a relatively higher extension.

Stress–Strain Relation

Figure 6 shows Mooney–Rivlin plot for various swollen collagen fibers according to eq. (6)[24,25]:

$$\phi = C_1 + C_2/\lambda \tag{6}$$

where the quantity ϕ is defined as $fv_2^{-1/3}/2(\lambda - \lambda^{-2})$; f, as the force per unit unstrained swollen area; v_2, as the volume fraction of rubber in swollen state; λ, as the strain ratio; and C_1 and C_2, as the constants.

The deviations from linearity with increasing strain (decreasing $1/\lambda$) represent the non-Gaussian region of strain. Even for the crosslinked RTT2 collagen fibers, an approximately linear relation is obtained in the initial strain range and parallel to the $1/\lambda$ axis. This means that $C_2 = 0$ and suggests that the retractive forces arising from the swollen network are mainly entropic, which is the same conclusion as derived from force–temperature relationships. At higher strains, the upturn takes place from the straight line. It is supposed, therefore, that in the initial extension regions the Gaussian statistics in the rubber network can be applied for the swollen collagens either in the 8*M* LiBr–BC or the 8*M* LiBr–MC system. The ϕ value in the initial extension regions at $C_2 = 0$, which is equal to C_1 as a function of the network structure, is analogous to one-half the value of shear modulus, G, in the statistical theory. As compared with the ϕ value for the RTT2 collagen crosslinked with BVSP in both systems,

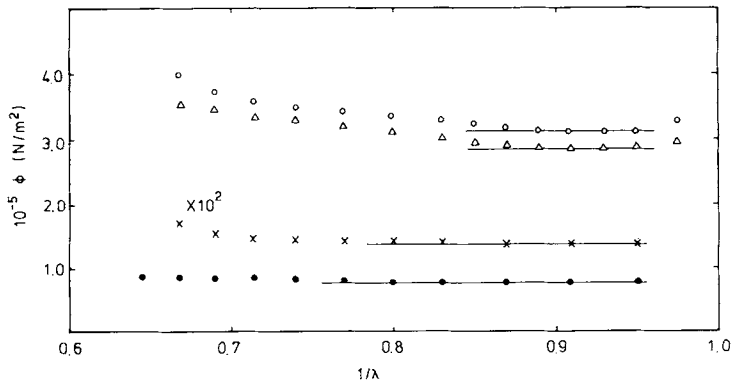


Figure 6 Mooney–Rivlin plot for various collagen fibers: (●) whale ligament; (×) rat-tail tendon (RTT2); (○) BVSP crosslinked rat-tail tendon (RTT2) in 8*M* LiBr–BC; (△) BVSP crosslinked rat-tail tendon (RTT2) in 8*M* LiBr–MC.

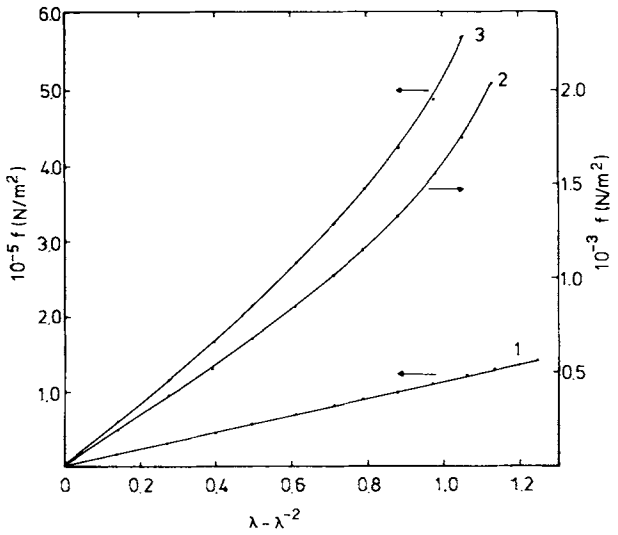


Figure 7 Relationships of equilibrium forces, f, vs. $\lambda - \lambda^{-2}$: (1) whale ligament; (2) rat-tail tendon (RTT2); (3) rat-tail tendon (RTT2) crosslinked with BVSP. $f - \lambda$ relation was measured in 8*M* LiBr–BC solution.

the quantity ϕ is almost the same, suggesting that both diluent systems are useful for the estimation of the shear modulus, which is directly related to the number of crosslinkages in collagen fibers. The ϕ values for whale ligament and uncrosslinked RTT2 collagen are considerably less than for the crosslinked collagen and the linear relation is continued to a higher strain range.

Determination of the Number of Crosslinkages

The crosslink density of the Gaussian network or the number of chains in unit volume of dry polymer, ρ/M_c, can be calculated by eq. (7)[24,26]:

$$f = Gv_2^{1/3}(\lambda - \lambda^{-2}) \tag{7}$$

where f is the equilibrium stress referred to the swollen cross-sectional area; λ, the strain ratio; v_2, the volume fraction of the polymer in the swollen sample; and G, the shear modulus of the swollen sample. Here, G is represented by eq. (8):

$$G = (\rho RT/M_c) \tag{8}$$

where R is the gas constant; T, the absolute temperature; ρ, the density of dry sample; and M_c, the number-average molecular weight between crosslinkages.

According to eq. (7), the crosslink density, ρ/M_c, was calculated from the initial slope of f versus the $(\lambda - \lambda^{-2})$ plot shown in Figure 7. The number of crosslinkage points per α chain of the collagen molecule, n, is expressed as $n = 10^5/M_c$. Here, the molecular weight of the chain was assumed to be 10^5. The values are shown in the last column in Table III. The imperfect network structure of RTT2 collagen is clearly suggested. Interchain crosslinkages between the chains of the same tropocollagen molecule consisting of triple chains could not be detected mechanically if no crosslinkages occur between the tropocollagen molecules. A complex situation, therefore, arises in order to study the relationship between the number of crosslinks and the crosslinking structure of collagen since the intramolecular crosslinks of the same tropocollagen molecule act as mechanically active crosslinkages when the intermolecular crosslinkages are introduced between tropocollagens.

It has been reported that most collagen crosslinking results from enzyme-mediated reactions

Table III Results Obtained from Shear Modulus Measured at 25°C in 8*M* LiBr–BC and 8*M* LiBr–MC Diluents

Collagen Samples	ρ (g/cm^3)	Diluents	v_2	$10^{-3}\,G$ (N/m^2)	$10^6\,\rho/M_c$ (mol/cm^3)	$10^{-4}\,M_c$ (g/mol)	n[a] (mol)
Rat-tail tendon							
RTT 2	1.30	LiBr–BC	0.100	2.91	1.18	110	0.09
RTT 10	1.30	LiBr–BC	0.118	129	52.1	2.5	4.0
Whale ligament	1.32	LiBr–BC	0.290	172	69.4	1.9	5.3
Crosslinked with BVSP							
RTT 2	1.33	LiBr–BC	0.298	622	250	0.53	18.9
RTT 2	1.34	LiBr–MC	0.257	602	244	0.55	18.2

[a] The number of crosslinking sites per collagen chain with average molecular weight of 10^5 and the value calculated as $10^5/M_c$.

Table IV Amino Acid Compositions of BVSP-treated and Untreated Rat-tail Tendon

	Tendon		Crosslinked Tendon	
Amino Acids	μmol/g	Res.[a]/1000 Res.	μmol/g	Res./1000 Res.
Lys	320	37	135	17
Arg	543	62	429	54
Asp	400	46	356	45
Thr	166	20	203	25
Ser	463	53	485	60
Glu	766	88	774	97
Pro	1329	152	1342	168
Hyp	749	86	697	87
Gly	2126	242	1910	237
Ala	1129	129	971	121
Val	340	39	358	45
Met	97	11	45	6
Ileu	106	12	103	13
Tyr	46	5	58	7
Phe	160	18	142	18
Total	8740	1000	8008	1000

[a] Res., residues.

involving the amino groups of lysine (Lys), hydroxyl lysine (Hlys), and arginine (Arg) residues located in the N- and C-terminal nonhelical regions of tropocollagen.[9,27] A recent study also indicated the existence of helix–helix collagen crosslinks in Type I skin fibers.[28] In both collagens, RTT10 and whale ligament, a three-dimensional network is developed in a similar order of crosslinkages. The number of crosslinkage points reaches about four to five per chain, suggesting the occurrences of the crosslinkages between tropocollagens.

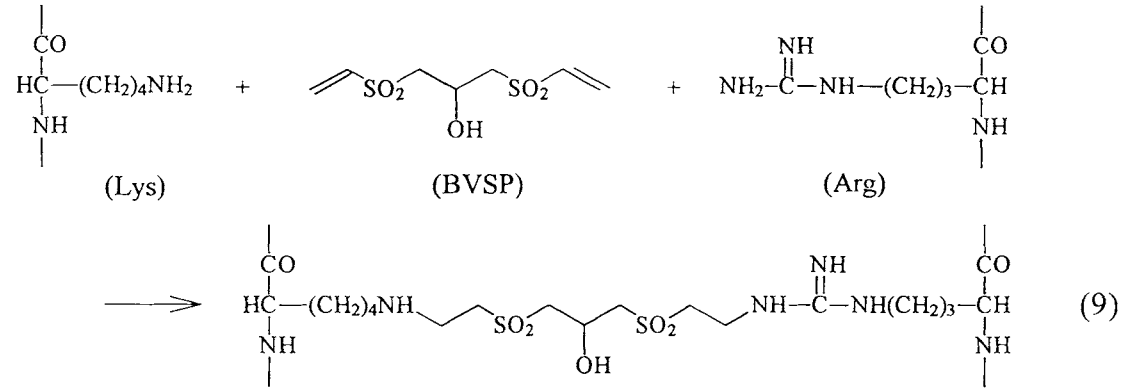
Difunctional BVSP gives about 18 mol per α chain as the number of crosslinkage sites. This calculation shows that there are many crosslinking sites between tropocollagen molecules being arranged in a staggered longitudinal direction. The distribution of the crosslinkage sites, which reflects possibly the distribution of Lys and Arg residues, is expected not to be random. In Type I collagen, the –Lys–Arg–sequence and short repeats of these amino acids have been found.[29] When crosslinking sites occur on closed positions of these residues, the network chain between the crosslink points seems unlikely to behave as a random chain. Accordingly, the crosslinkages introduced are expected to be too near to differentiate them as different crosslinkages using the present mechanical method based on the deformation of network. It is supposed, therefore, that the number of actually introduced crosslinks may be larger than the calculation.

With regard to the shear modulus, G in eq. (8), the loose end correction term, $(1 - 2M_c/M)$, which would be used for an usual randomly crosslinked network[30] made up from primary molecules with average molecular weight of M, needs not to be introduced for the collagen network crosslinked with BVSP in which loose ends are likely to be absent. It is appropriate to consider that the rod-like tropocollagen molecules are crosslinked with each other through about 54 sites to the neighboring molecules.

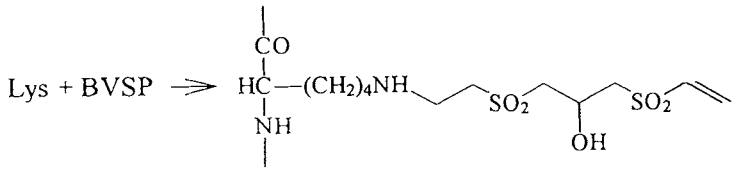
Number, Type, and Site of Crosslinks in Collagen Treated with BVSP

Table IV shows the amino acid compositions of BVSP-treated and untreated RTT2 collagen fibers. The contents of Lys and Arg in the treated fibers are decreased, especially the Lys content. The other amino acids seem to be unchanged in their content within experimental error. The total amount of Lys and Arg residues lost by the reaction with BVSP is about 300 and 280 μmol/g on the basis of the content in residues/1000 residues. The number of residues associated with the

sites of interchain crosslinkages was about 180 μmol/g ($= n \cdot 10^6/10^5$), which corresponds to about 60–64% of the reacted amino groups. As described in the preceding discussion, the number of actually introduced crosslinkages may be larger than this. The other amino groups probably exist as either the sites of mechanically ineffective intrachain crosslinkages or pendant groups involving unreacted vinyl groups:



(9)



(10)

The reaction of Lys and Arg residues with BVSP is shown in eq. (9). Interchain and intrachain crosslinkages may be formed between the residues of Lys and Arg, between Lys and Lys, and between Arg and Arg. As shown in eq. (10), pendent groups may be also formed on Lys and Arg residues located on the peptide chain with a lack of the juxtaposed Lys and Arg residues existing in sterically favored conformations for the reaction with BVSP.

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