

ANALYSIS OF THE EFFECTS OF pH AND TENSILE DEFORMATION ON THE SMALL-DEFORMATION MODULUS OF CALF SKIN

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A dynamic technique was used to measure the elastic modulus of the reticular dermis of calf skin at a small (0.1%), non-perturbing oscillating deformation. The elastic modulus increases by over an order of magnitude when the tissue is stretched by only 10% before the measurement is made. The results are interpreted by a stochastic model of fiber orientation that includes the orientation of fibers and their straightening out from slack configurations into the load-bearing structure. An equation is derived that accurately describes the course of the modulus-extension curve over a range of pH from 3 to 11, and allows the calculations of the properties and configurations of the slack fibers. One of these is the Young's modulus of a fiber, which agrees well with the previous determinations in neutral medium.

KEYWORDS: Collagen, skin, fibers orientation(s), dynamic modulus, mechanical properties, fibrous, swelling.

INTRODUCTION

Although understood in qualitative terms, the resistance of skin to large deformation has not successfully been analyzed. Superficially the structure of skin resembles a fiber-reinforced engineering composite, since the fibers are very long, with a distribution of orientations that can be described statistically, imbedded in a continuous matrix. An important difference from such composites, however, is that in skin the matrix is very compliant and apparently¹ not essential for transferring stress from one fiber to another. As a result, only fibers that are extended between intersections of a load-bearing network actually contribute to the modulus. In unstretched skin these fibers are only a few. The need for a more appropriate approach was understood by Soong and Huang,² who introduced a stochastic model for the fiber network. This model was a point of departure for the sophisticated and rigorous analysis of Lanir,³ who related the numbers of straightened, load-bearing, fibers to the changing geometry of the skin while it is subject to an arbitrary biaxial deformation. From this analysis he was able to calculate stress-strain curves with the characteristic shape of that of skin and showed how information about the types of fibers and the frequency distribution of their orientations could be obtained from them.

In the Lanir model of skin, collagen fibers are first straightened and then stretched homogeneously, following the macroscopic deformation. This describes

a system in which some fibers that are initially almost straight, which determine the initial part of the stress-strain curve, are stretched elastically up to 100% before the sample breaks. Such extensions, however, exceed the elastic limit of collagen fibrils. In the examples presented,³ half the fibers are load-bearing at the “ankle” of the stress-strain curve; some, having been stretched 60%, are about to be extended another 20% at deformations beyond the ankle. The basic collagen fibrils from tendon, similar to those that comprise the structure of skin, have been observed by electron microscopy and by X-ray diffraction under deformation. Extensive disruption occurs at only 4% extension of the tendons, with loss of longitudinal order by intrafibrillar shear,⁴ and with longitudinal yielding at extensions above 20%.⁵ Certainly the fibers cannot be expected to behave elastically over any practical range of deformation.

If the fibers or fibrils were to slide with respect to each other at stresses that are smaller than those required for the collagen fibrils to yield internally, they would become oriented in the tensile direction during the deformation while only slowly increasing the external load. We have observed on the other hand that the incremental stress associated with a small incremental deformation, applied and removed within 1 s, is not appreciably relaxed by the sliding process, which takes longer. This contributes to the well-known⁶ lack of correspondence between the incremental modulus of a biological sample, determined rapidly while the sample is being progressively stretched slowly, and the slope of the stress-strain curve. Therefore, the low-deformation modulus can be analytically related to orientation of the straightened fibers, even if the total force cannot. Further, if the orientation of the fibers can be expressed in terms of the elongation of the sample, an equation can be derived relating the strain to the incremental modulus instead of to the stress.

THEORY

We adapt the structural model developed by Lanir³ to a calculation of the incremental modulus of fibrous tissue under deformation. That is to say that fibers contribute to the modulus as they are straightened and enter the active network, even though, because of slippage, they do not continue to stretch elastically. In our treatment, the whole network is considered to comprise undulating collagen fibers, which are bundles of mutually aligned collagen fibrils. This undulation is considered to be inherent and not necessarily induced by elastin, which our model ignores. We treat first a single homogeneous class of collagen fibers; then generalize to a distribution of fibers with different geometries in equation (5).

The fibers have undulating segments between points at which they are entangled or attached to each other. Let

l_0 = length of the end-to-end vector of such segment in the undeformed tissue;

l_s = length of one of these vectors in a segment that has just been pulled straight but not yet stretched;

λ = elongation ratio of the sample;

λ_s = elongation ratio l_s/l_0 required to just straighten a segment;

θ = angle of the end-to-end vector of a segment with tensile axis;

E_m = elastic modulus of a segment (measured along its end-to-end vector) stretched beyond λ_s but still within its elastic limit. It is equivalent to Young's modulus of a straight collagen fiber.

When the tissue is stretched, the fibers are extended and rotated. As described by Lanir,³ the elastic force on each straightened segment lying at angle θ to the tensile direction is

$$F(\theta) = E_m(\lambda(\theta) - \lambda_s)/\lambda_s, \quad (1)$$

where $\lambda(\theta)$ is the stretch ratio of a segment oriented at angle θ . From the empirical observation that there is no density change on stretching, the elongation ratios $\lambda_x, \lambda_y, \lambda_z$ in the principal directions of the sample x, y, z relate to λ by the affine transformation

$$\begin{aligned} \lambda_x &= \lambda_y = \lambda^{-1/2} \\ \lambda_z &= \lambda. \end{aligned} \quad (2)$$

In any longitudinal plane, from equation (13) of reference (3),

$$\lambda(\theta) = (\lambda_z^2 \cos^2 \theta + \lambda_x^2 \sin^2 \theta)^{1/2} = (\lambda^2 \cos^2 \theta + \lambda^{-1} \sin^2 \theta)^{1/2}. \quad (3)$$

Note that $\lambda(\theta)$ is maximal at $\theta = 0$ and becomes less than 1 at large angles (vide infra).

The sample in our experiment has a small oscillating tensile deformation superposed on the much larger static deformation. The corresponding differential force is measured to determine the small-deformation modulus, E' . The incremental tension in a straightened segment lying at angle θ from the tensile direction that contributes to this incremental modulus is $E_s(\theta)$:

$$E_s(\theta) = dF(\theta)/d\lambda = \frac{E_m(2\lambda \cos^2 \theta - \lambda^{-2} \sin^2 \theta)}{2\lambda_s(\lambda^2 \cos^2 \theta + \lambda^{-1} \sin^2 \theta)^{1/2}} \quad (4)$$

Let $E(\theta)$ be the modulus (measured in the tensile direction) of the assembly of segments that have been straightened and are lying at angle θ . These have relative elongations $\lambda(\theta)/\lambda_s$. Their number depends on the frequency distribution of λ_s , $P(\lambda_s)$, and on whether λ_s is smaller than the elongation imposed on a segment. Therefore we integrate the contributions of the straightened segments lying about angle θ , equation (4), over values of λ_s that are smaller than $\lambda(\theta)$:

$$E(\theta) = \frac{E_m}{2} \int_1^{\lambda(\theta)} \frac{P(x)(2\lambda \cos^2 \theta - \lambda^{-2} \sin^2 \theta) dx}{x(\lambda^2 \cos^2 \theta + \lambda^{-1} \sin^2 \theta)^{1/2}} \quad (5)$$

The modulus of the sample is the sum over the contributions of straightened segments lying about all angles θ . At large θ , however, the segments are compressed rather than extended because of the contractions in x and y described in equations (3); the angle at which this first occurs is θ_c :

$$\theta_c = \tan^{-1} [\lambda(\lambda + 1)]^{1/2} \quad (6)$$

The resultant modulus is obtained by integrating $E(\theta)$ in equation (5) over θ , with proper consideration to the vectorial properties of $E(\theta)$. Since segments lying at angles greater than θ_c do not contribute to the modulus, the limits of the integration are $-\theta_c$ and θ_c . If the starting distribution of orientation angles of segments is $R(\theta)$,

$$E' = \int_{-\theta_c}^{\theta_c} \frac{E(\theta) R(\theta) \cos^2 \theta \, d\theta}{\lambda(\theta)} \quad (7)$$

Equation (7) is an application of the general equation (45) of reference (3). The cosine-squared factor results from a product of the component of force in direction θ times the component of unit area perpendicular to the fibers that lies in the sample cross section, over which fibers are counted. The $\lambda(\theta)$ factor is part of the expression that accounts for rotation of the fibers, which is derived from equation (19) of reference (3).

To apply equation (7) to bovine hide, we have assumed a Gaussian frequency distribution of λ_s :

$$P(\lambda_s) = (1/\sigma \sqrt{2\pi}) \exp [-(\lambda_s - \lambda_{av})^2/2\sigma^2], \quad (8)$$

where λ_{av} is the mean value of λ_s and σ is the width of the distribution. In addition, bovine hide is a full three-dimensional network of fibers, so the angular distribution function $R(\theta)$ must be weighted with a factor $2\pi \sin \theta$ but must be normalized over only a single hemisphere to avoid counting segments twice. We have observed no consistent preferred orientation in our samples before they were stretched, so

$$R(\theta) = (2\pi \sin \theta)/2\pi = \sin \theta. \quad (9)$$

Equations (7), (8), and (9) give:

$$E' = \frac{E_m}{\sigma \sqrt{2\pi}} \int_0^{\theta_c} \frac{(2\lambda \cos^2 \theta - \lambda^{-2} \sin^2 \theta) \cos^2 \theta}{(\lambda^2 \cos^2 \theta + \lambda^{-1} \sin^2 \theta)} \int_1^{\lambda(\theta)} \frac{\exp[-(x - \lambda_{av})^2/2\sigma^2] dx d\theta}{x} \quad (10)$$

for the incremental modulus E' as a function of λ . For the case of planar deformation,

$$E' = \frac{E_m}{\sigma \pi \sqrt{2\pi}} \int_0^{\theta_c} \frac{(2\lambda \cos^2 \theta - \lambda^{-2} \sin^2 \theta) \cos^2 \theta}{(\lambda^2 \cos^2 \theta + \lambda^{-1} \sin^2 \theta)} \int_1^{\lambda(\theta)} \frac{\exp[-(x - \lambda_{av})^2/2\sigma^2] dx d\theta}{x} \quad (11)$$

METHODS

Fresh-frozen calfskin specimens. A calf skin was refrigerated on ice immediately after slaughter, cleaned, and frozen within 5 hr. The reticular dermis was isolated by removing the distal and proximal layers with a leather splitting machine (Fortuna

Werke AG*, Stuttgart), which uses a moving band knife opposed to a pair of rollers which hold the semi-frozen skin firmly. In this state, deformation of the sample by the rollers is minimized, but it might affect the initial orientations of the fibers as mentioned in Results. The skin was thus thinned to 1 mm and then cut into $4 \times 0.5 \text{ cm}^2$ strips. Copper tabs were glued to the two surfaces of the strips near each end with cyanoacrylate glue to avoid deforming the specimens in the clamps of the tensile-measurement instruments.

Each specimen was equilibrated with medium for 2 hr, after which the dynamic modulus became constant even for the samples at the extremes of pH. The various media were buffered with 0.05 M phosphate at pH of 6, 7, and 8; 0.05 M sodium acetate at 4 and 5; 0.05 M Tris at 9; and 0.05 M sodium carbonate at 10 and 11. The medium at pH = 3 was unbuffered but contained 0.05 M sodium chloride.

Extracted calfskin. Non-collagenous material was removed from the skin used to compare the tangent and dynamic moduli by a process usually used to prepare skins for tanning.⁷ It was agitated for 2 hr at 29° in 2% sodium hydrosulfide and saturated calcium hydroxide and 20 hr at 27° in 0.5% sodium hydrosulfide and saturated calcium hydroxide; after having been washed it was neutralized with ammonium sulfate to a pH of 9.0, and then to a pH of 7.0 with 0.1 M potassium phosphate.

Mechanical measurements. Stress vs strain was measured by means of an Instron Universal Tester with metal gauze covering the 6 cm^2 surfaces of the grips. The wet extracted samples, measuring $2.5 \times 0.6 \times 0.3 \text{ cm}^3$ between the clamps, were elongated at 17%/s in room air.

The incremental modulus can be measured most simply with a conventional stress-strain tester by applying small loading-unloading cycles to a stretched sample.⁶ The slopes of the small stress-strain curves obtained approximate the incremental moduli at increasing strains. This method is not very precise, however, because only a small part of the range of the stress transducer is utilized. As a result, the measurement cannot be performed at very small incremental strains, less than about 0.02. For greater precision and smaller incremental deformations a vibratory measurement is preferred. The sample can be mounted as in the previous apparatus, but repeated small stress-strain cycles are applied, and the root-mean-square average stress and strain are determined and are used to compute the elastic modulus. The stress transducer can be compensated for the static component of stress, so that the small alternating component can be measured with the full sensitivity of the instrument. The method can be used for samples with irregular shapes, such as are met in *in-vivo* studies.^{8,9} A full explanation of the method and its application to skin with a commercial instrument has been published previously, although with no mention of the effects of superposed static stretching.¹⁰ The incremental modulus can also be calculated from the velocity of sound in skin. The resulting "sonic modulus" corresponds to even smaller deformations than usually applied in the other two methods, and the value is very precise. The method would probably be preferred in cases where the

*Reference to a brand or firm name does not constitute endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.

sample geometry and the mode of wave propagation are simple. The sonic modulus has been related to molecular orientation in anisotropic samples of synthetic polymers.¹¹

We determined the incremental modulus on fresh-frozen calfskin by the vibratory method with a Rheovibron DDVII viscoelastometer (Toyo-Baldwin Co. Inc.,* Tokyo) set for a differential elongation of approximately 0.0015 and a frequency of 110 Hz. The advantages of this instrument¹² are convenient sample changing, high precision, small ($\geq 13 \mu\text{m}$) but easily variable vibratory elongation, and frequency variable from 0.001 Hz to 110 Hz. The device imposes a sinusoidal deformation at constant frequency to a small tensile sample and determines the accompanying oscillating stress and its phase with respect to the deformation. It allows a precise determination of the components of $E^* = E' + E''$, the complex modulus of skin.¹⁰ In our measurements it was found that E'' , the loss modulus, was less than 10% of E' , the elastic modulus, so our data treatment could be applied to either E' or to $(E'^2 + E''^2)^{1/2}$, the magnitude of E^* . One of the sample clamps can be moved to precisely set the static length of the sample while measuring the static (average) force or the dynamic (incremental) force, so that the incremental modulus can be obtained as the sample is elongated, point by point.

The sample was immersed in a recirculating bath at constant temperature¹³ containing the buffered medium. At the start of an experiment a specimen would be clamped in the bath in a slack condition for 30 min; then a force of 0.01 N would be applied, which was just sufficient to straighten it. The dynamic modulus was determined at increasing steps of static elongation, the sample being allowed to relax at zero force for 3 min between each because it was found that this relaxation improved the reproducibility.

After an experiment the sample was cut from its copper tabs, blotted and weighed, washed with water, dried *in vacuo* at 110° for 2 hr, and weighed again. The modulus was calculated on the basis of the area of dry material in the cross section, determined from the mass and initial length by the formula $(\text{mass})/(\text{initial length} \times \text{density of dry collagen}) = (\text{area})$. This method of calculating modulus eliminates the purely dimensional effect of sample swelling at extremes of pH, preventing it from confounding observations of chemical effects on the load-bearing substance. Both the lengths and cross sections were the actual values at each static elongation; for the cross section we assumed a constant density of 1.35 g/cm³.

Equations 10 and 12 were fitted to experimental data by the Gauss-Newton iteration technique,¹⁴ using a Modcomp Classic 7861 computer with the program ABACUS D.2 developed by the ERRC computer center. Data from the Instron instrument was digitized from a recorder chart by means of a digitizing pad.

RESULTS

Comparison of E' with tangent of stress-strain curve. In order to compare the two quantities, each of which varies over a large range, we have plotted their logarithms vs elongation in Fig. 1. While both measures of modulus increase more than

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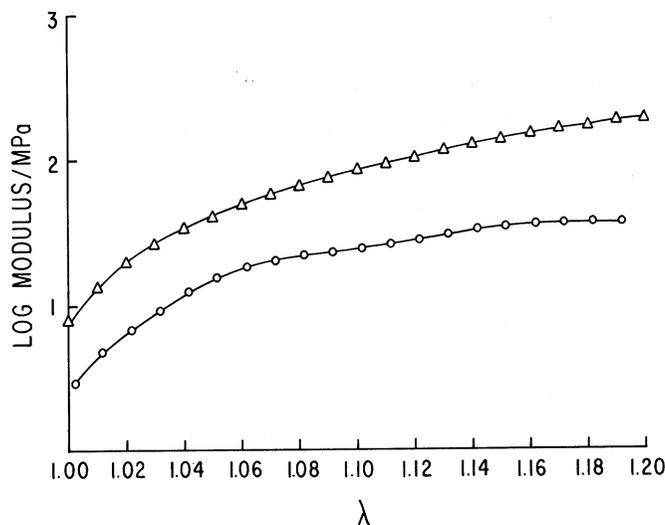


FIGURE 1 Comparison of the dynamic elastic modulus, E' , with the tangent to the stress-strain curve of extracted calf skin, as a function of tensile elongation. Δ , E' ; \circ , tangent modulus, E_{tan} .

ten fold with elongation, E' is persistently at least 4.9 times larger than the tangent modulus. The behavior shown in Fig. 1 is typical; experimental values of E_{tan} and of E' at two elongations are given in Table I.

We fitted the E' -elongation data, obtained at pH from 3 to 11, to equation (10) to obtain the parameters E_m , λ_{av} , and σ at each pH. The values of E_m are shown in Fig. 2a. Since E_m is intrinsic to the collagen fibrils, it should not be sensitive to textural or configurational variations in the fibers due, for example, to handling. Thus expecting that it should be a smooth function of pH, we fitted a quadratic curve to the data which is shown in Fig. 2a:

$$E_m = -35.4 + 15.9(\text{pH}) - 1.1(\text{pH})^2 \quad (12)$$

The water content in our samples is shown for comparison with E_m in Fig. 2b. The opposite behavior might be expected from the previous observation of Bowes and Kenton,¹⁵ but, unlike theirs, our values of modulus have been corrected to a dry collagen basis. The large residual variation of E_m with pH is then due to the varying intermolecular and interfibrillar interactions, but not to the dilution of the collagen.

TABLE I

Comparison of average dynamic and tangent moduli of extracted calf skin as described in Methods. E' values are reported at relative elongations λ of 1.01 and 1.12; E_{tan} , at 1.00 (extrapolated) and 1.12.

Elongation	E_{tan} (MPa) (n=4)	E' (MPa) (n=3)
1.0	1.45 ± 0.28	12.5 ± 3.9
1.12	22.5 ± 7.2	121 ± 29

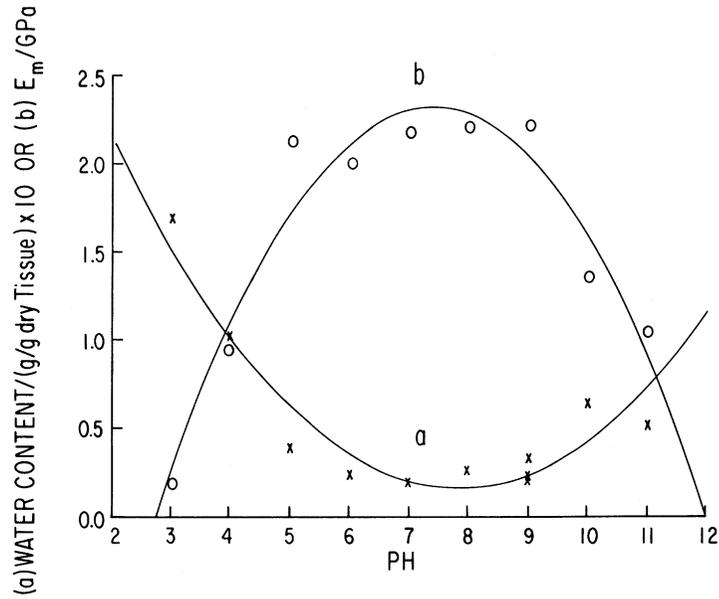


FIGURE 2 Behavior of Young's modulus of the collagen fibers of calf skin reticular dermis, E_m , in media of various pH (\circ), compared to that of the water content of the tissue (\times).

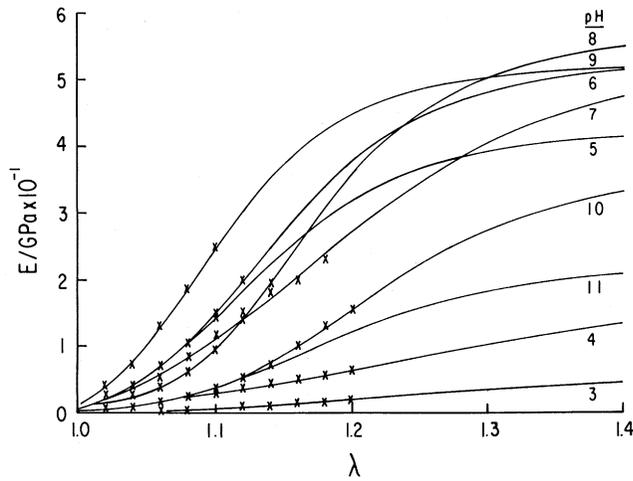


FIGURE 3 E' of calf skin reticular dermis as a function of elongation at various values of pH, with functions calculated from equation (10) in the text, using adjusted constants E_m , λ , and σ .

The complete E' -elongation data are shown in Fig. 3, along with the theoretical curves refitted with the smoothed values of E_m from equation (12). Fig. 3 and the RMS deviations in Table II show that the functions fit very well. In fact these RMS deviations changed very little when the smoothed E_m values were substituted and were held constant with optimization of λ_{av} and σ . It is unfortunate that the experimental data cannot be extended to the plateau regions of the theoretical

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TABLE II

Fiber parameters of calf skin deformation at various values of pH and statistical deviation of experimental points from curves in Fig. 2.

pH	E_m (GPa)	λ_{av}	σ	RMS deviation
3.0	2.5	1.21	0.11	0.0068
4.0	10.9	1.23	0.19	0.0076
5.0	17.1	1.10	0.06	0.0084
6.0	21.2	1.11	0.06	0.0169
7.0	23.1	1.14	0.10	0.0700
8.0	22.8	1.13	0.05	0.0261
9.0	20.4	1.07	0.05	0.0380
10.0	15.9	1.17	0.07	0.0191
11.0	9.76	1.14	0.07	0.0157

TABLE III

Variability of fiber-undulation parameters of calf skin among six different samples.

pH	λ_{av}	σ
7.0	1.20	0.069
7.0	1.14	0.093
7.0	1.27	0.098
9.0	1.074	0.049
9.0	1.12	0.076
9.0	1.068	0.051

curves, but the experimental range is limited by the tensile strength of the skin and by the capacity of the Rheovibron instrument. Most of the samples could be extended to the point of inflection of their respective theoretical curves, at which point half the undulations of the collagen fibers are fully extended.

The shapes the individual curves are dominated by fiber straightening described by the inner integral of eq. (10), not by the rotations of segments. They are sensitive to sample handling as well as to pH. The difficulty of defining the undeformed initial state of soft tissue is well known. In this experiment E' is measurable even in slack samples. It was therefore expected that the behaviors of λ_{av} and σ would be somewhat idiosyncratic, since they are sensitive to initial straightening of fibers due, for example, to squeezing of the sample when it is cut and shaped, however carefully. The variations (at constant E_m) for triplicate measurements at pH=7 and 9, shown in Table III, bear this out. Table II, however, shows no systematic change with pH.

DISCUSSION

Although both the tangent modulus and E_m increase with strain, their magnitudes are very different (Fig. 1). This difference has not been fully explained, but has been discussed previously.⁶ The materials in the two experiments have undergone different strain histories, the tangent modulus being determined retrospectively,

and may encounter different elongation-dependent relaxation processes. The observation extends to both extracted and untreated samples; extraction doesn't affect the moduli noticeably, as can be inferred by comparing the data of Table I with that of Fig. 3.

The model that we propose for the stretching of skin accepts that fibers slip after they have been straightened, but assumes that they slide relatively less during very small, rapidly oscillating deformations than in large, steadily increasing ones. The principal effect of this approximation is expected to be a lowering of E_m and of the calculated modulus at the plateau. A smaller effect, on the final distribution of fiber orientation angles, would not be important in our experiments since they were carried to only about 20% elongation.

When the swelling of cartilage is increased by a lowering of the ionic strength of the bathing solution, the type II collagen fibrils are straightened.¹⁶ We therefore might have expected that the enormous swelling of the skin at extremes of pH would have straightened the fibers, but such an effect is not evident in the values of λ_{av} and σ . This is evidence that the swelling of skin at extreme pH, unlike that of cartilage at low ionic strength, is a property of whole collagen fibers and is not restricted to a proteoglycan interfibrillary matrix.

Although data could not be obtained at elongations corresponding to the expected plateau of E' (Fig. 3), the validity of the level of this plateau can be inferred from the agreement of E_m with values obtained by other means, since the plateau is determined by E_m and the orientation distribution of the stretched sample. There are only few directly measured values of the modulus of collagen fibrils in the literature for comparison with our E_m , and these were only obtained in neutral media (Table IV).

The values of E in Table IV have been corrected for the presence of non-collagenous components in different ways. The amounts of collagen in the cross sections of the samples used in the stress-strain measurements were only roughly estimated, so these moduli on the basis of collagen could vary between 1.0 and 3.5 GPa. The Brillouin calculation¹⁸ did not have to be corrected for the amount of collagen in a cross section, but did require estimates of the transverse and shear moduli, which could easily have given an error of 50%. Our value, as explained, is essentially an extrapolation. The four values therefore agree within their estimated approximations. The Brillouin value will be capable of later refinement if the lacking elastic constants become available. It is expected to be higher than the other values because of the higher frequency (ca. 10 GHz) and the fact that

TABLE IV
Young's Modulus of Collagen Fibers at Neutral pH

E (GPa)	Method	Reference
3.0	Mechanical stress-strain curve, tendon fibers.	15
1.4	Mechanical stress-strain curve, bovine skin fibers.	16
5.1	Brillouin scattering (phonon velocity), tendon fibers.	17
2.3	Small oscillations, bovine skin.	Eq. 11

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the technique samples volumes on the scale of individual fibrils, so that inter-fibrillar movement does not contribute to the deformation. The variation of E_m with pH is much greater than the differences in Table IV.

The value of E_m might be lower for skin than for tendon because of differences in the structures of the fibrils themselves. As shown by small-angle X-ray scattering of tendon during deformation,¹⁹ fibrillar deformation includes both molecular and intermolecular modes. The long spacing of demineralized bone collagen fibrils²⁰ and of tendon fibrils²¹ are 67 nm, whereas that of skin fibrils is only 65 nm.²¹ The shorter period in skin fibrils has been attributed to a more helical and presumably more compliant configuration.

Recognizing that fibers can slip past each other after they have been pulled straight, we find that the stochastic model still accurately describes the increase in incremental modulus of stretched calf skin, even if it fails in calculations of total force. It provides a parameter, E_m , which agrees with the modulus of collagen fibers determined by other means in neutral media, and has permitted a convenient determination of this modulus for the first time over a wide range of pH, from 3 to 11. The behavior indicates effects of electrostatic interactions or disruption of labile crosslinks on the mechanical properties of collagen in the solid state that are independent of geometrical effects of swelling.

Although equation (10) cannot be integrated to give the stress, the low-deformation modulus $E'(\lambda)$ is useful for describing the response to small incremental strains of stretched soft fibrous tissue such as blood vessel walls, skin, intestinal wall, or tendons. Three-dimensional strain is appropriate for bovine hide, but equation (11), describing planar deformation with or without initial anisotropy, would probably better fit human skin. On the other hand, this analysis does not apply to dry soft tissue or bone, which have been successfully treated by the methods suitable for composites.^{22,23}

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