

P. L. Kronick

M. S. Sacks¹

U.S. Department of Agriculture, ARS,
Eastern Regional Research Center,
Philadelphia, PA 19118

The chemical basis of viscoelasticity of bovine skin was explored by mechanical relaxation spectroscopy after selective enzymatic degradation. Measurements covered a wide range of time scales because water was replaced in the tissue with aqueous mixtures of ethylene glycol, which maintained a water-like electrical environment for the charged macromolecules down to -50°C . Macromolecular components that couple the fibrils to the interfibrillar matrix contribute about half the values of the resultant storage and loss moduli, while removal of components that are readily extractable, so perhaps free in the matrix, did not alter these mechanical quantities or their relaxations. The precision of the method reveals the effects of fibril-attached matrix, when conventional methods of mechanical testing fail.

Introduction

The viscoelasticity of soft tissues is an essential aspect of their mechanical properties. Its importance in biomechanics has appeared mainly in introducing time dependence into phenomenological stress and strain responses. The viscoelasticity of synthetic gels, however, has been successfully related theoretically to the molecular structure in the last few years. The success of these investigations depended on the freedom to vary temperature and frequency over wide ranges [1]. Application to tissue requires the ability to go far beyond the temperature limits of the freezing of water and denaturation of proteins (0°C to 40°C). Obviously a water substitute is required that mimics water in ways essential to preservation of molecular configurations and allows a wide variation in temperature; i.e., a hydromimetic solvent.

Low-temperature studies of enzyme kinetics in alcoholic solvents offer one possibility for a hydromimetic solvent. We assume that soft connective tissue can be modelled as fibers imbedded in a gel matrix, which is made viscoelastic by proteins and proteoglycans. The configurations and the states of aggregation of these polyelectrolyte molecules in tissue depend on the ionic strength and the dielectric constant of the fluid medium in the matrix. It has been shown that the configurations of proteins remain native in aqueous ethylene glycol mixtures as long as the temperature is lowered enough to hold the dielectric constant at 80 [2]. Thus many enzymatic activities are preserved (although the reactions are thermally slowed) and details of structure as determined by X-ray diffraction are unaltered, except for more restricted movements of sidechains.

To study relationships between mechanical properties and structure, selective removal by enzymatic attack on the components of connective tissue has been used in the past with mixed results. One difficulty in determining the contributions of these components to the mechanical properties has been the

great variability among tissue specimens, making it almost impossible to study effects of the enzymes when they are small. Partington and Wood [3] found that the elastic modulus of tendon, if measured at small deformation, could be measured repeatedly on the same specimen, so that a single specimen could serve as its own control. They report that removal of hyaluronic acid had no effect on the stress-strain curve, but that removal of an unidentified noncollagenous protein decreased the 2 percent modulus and the tensile strength. Dale [4] also found that treatment with trypsin affected the mechanical properties of tendon, by removing a component that stabilized the fiber crimp. Oxlund and Andreassen [5], however, found that treatment with hyaluronidase or with α -amylase had no effect on the stress-strain curve of rat skin, and Viidik et al. [6] found no statistically significant effect of enzymatic removal of either hyaluronic acid or dermatan sulfate on the stress-strain curves of rat-tail tendons. Thus, effects of enzymolysis still remain uncertain.

In the study described here, we applied a very small oscillating deformation while measuring the force. Measurement of the modulus repeatedly at a small dynamic strain ("dynamic modulus") avoids the problem of the poor reproducibility of stress-strain curves from which mechanical properties were previously derived and also gives a complete description of viscoelastic behavior. Further, we considered that the interfibrillar material was likely to affect the viscosity component of the viscoelasticity, so we measured the dynamic modulus over a wide range of times and temperatures. This was achieved by replacing the water of the tissue with 50 or 70 percent ethylene glycol, which are hydromimetic in the sense discussed above at temperatures around -50°C [2]. This method had the further advantage of a reproducible initial state of strain because the modified skin was relatively stiff at room temperature.

Methods

Materials. Two calfskins were obtained at different times during the study from freshly slaughtered 6-month old animals. The first was used for the salt-extraction study; the second for

¹Present address: Department of Biomedical Engineering, University of Miami Box 248294, Coral Gables, FL 33124-0621.

Contributed by the Bioengineering Division for publication in the JOURNAL OF BIOMECHANICAL ENGINEERING. Manuscript received by the Bioengineering Division November 20, 1990; revised manuscript received June 4, 1993. Associate Technical Editor: R. M. Hochmuth.

the enzyme studies. The following enzymes and their inhibitors were obtained from Sigma Chemical Co. Bovine testicular hyaluronidase was Sigma H3506; chondroitinase ABC (chondroitin ABC lyase, EC4.2.2.4) from *Proteus vulgaris*, Sigma C2905; bovine pancreatic trypsin (EC3.4.21.4), Sigma T8642; and trypsin inhibitor from soybean, Sigma T9003. Ethylene glycol was the analyzed 100 percent product from Baker Chemical Co.

Salt Extraction. A calfskin was frozen at -30°C within 3 hours after slaughter, allowing time for transportation, cleaning, scraping, and cutting into sections approximately $50 \times 50 \text{ cm}^2$. Two-millimeter thick layers of the reticular dermis of pieces from the butt, cut parallel to the backbone, were isolated by splitting them while partially frozen with a Fortuna (Stuttgart, Germany) leather splitter. Macromolecules, including hyaluronic acid, were twice extracted from half these samples by gently agitating them for 5 days at 10°C in a solution of 4.5 M NaCl, 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS), 20 mM ethylenedinitrilotetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM N-ethylmaleimide (NEM), and 1 g/ml pepstatin, the last four components being inhibitors of the proteolytic enzymes that might reside in the fresh tissue. High molecular weight material would cause an increase of the viscosity of the extracts, allowing us to determine the effect of the extractions. Viscosity measurements of the two extract solutions performed with an Ostwald viscometer showed the relative viscosity, $\eta_r = \eta_{\text{extract}}/\eta_{\text{water}}$, of the first extract solution to be 0.52; of the second, 0.10, indicating that a significant amount of high molecular weight material was removed with the two extractions, most of it with the first.

Treatment with Hyaluronidase. Calf reticular dermis isolated as described above was impregnated with enzyme by agitating gently at 4°C for 3 days in 1.5 units/ml [USP XXI-NF XVI] hyaluronidase in 0.1 M phosphate buffer at pH = 5.3 containing 0.5 percent NaN_3 , 20 mM EDTA, 1 mM PMSF, and 2 mM NEM. Digestion was then carried out by raising the temperature to 37°C and holding for 24 hr. Analysis by electrophoresis [7] showed that 99 percent of hyaluronic acid was removed, but only 29.8 percent of dermatan sulfate.

Treatment with Chondroitinase ABC. This enzyme degrades dermatan sulfate, chondroitin sulfate and hyaluronate to disaccharides [8]. Calf reticular dermis (10 g), isolated as described above, was impregnated with enzyme solution by gently stirring for 5 days at 4°C in 50 mM TRIS (pH = 7.4), 150 mM NaCl, 20 mM EDTA, 1 mM PMSF, 2 mM NEM, and 0.5 percent sodium azide. The treated sample was incubated for 5 hr at 37°C in solution containing 5 units chondroitinase ABC; the control sample solution had no enzyme. Analysis [9] showed that the uronic acid content of the treated sample was $0.1 (\pm 0.02)$ percent of collagen; that of the control, $0.2 (\pm 0.04)$ percent; indicating that 70 (± 14) percent was removed, after allowing for the interference with the assay due to 0.06 percent collagen-bound galactose or glucose. The collagen was determined by hydroxy proline analysis, assuming 13 percent hydroxyproline in collagen. The precision of the assay was ± 20 percent.

Treatment with Trypsin. Calfskin (10 g) was impregnated in 100 ml of 10 mM phosphate buffer, pH = 7.1, with 150 mM NaCl, 1 mM CaCl_2 , and 5 percent NaN_3 , containing 1 mg/ml trypsin. Control calf hide was prepared in the same solution with no trypsin. After the preparations were impregnated at 4°C for 4 days, they were incubated at 37°C for 1 hr. They were quenched at 4°C in a solution containing the inhibitors used for hyaluronidase. Analysis as above [9, 10] gave 0.1 percent proteoglycan in treated tissue; in control, 0.2 percent. We concluded that 70 (± 14) percent was removed,

allowing for 0.06 percent collagen-bound galactose or glucose, which interfere with the analysis.

Impregnation with Solvents. After having been treated as described above with individual enzymes, the specimens were rinsed with water and were impregnated with ethylene glycol-water. They were placed horizontally under water on a wire tray covering a magnetic stirrer at the bottom of a flat-bottom recrystallizing dish, while ethylene glycol dripped in over a period of 17 hr—slowly enough to prevent the sample from warping until the desired concentration was reached.

Measurements. We attempted to relate the loss modulus to the static viscosity of the GAG solutions of the impregnated interfibrillary gel by using GAG solutions as a model. The viscosities of supercooled ethylene glycol-water mixtures and of their GAG solutions were determined with a Brookfield rotary viscometer (Stoughton, MA), cooling stepwise with liquid nitrogen. There was no difficulty in supercooling (without crystallization) the 70 percent mixture to 193 K.

The complex modulus E^* of the tissue was measured by the method of forced oscillations with a Rheovibron DDVII viscoelastometer (Toyo-Baldwin, Tokyo) at 110 Hz, 11 Hz, and 3.5 Hz from -100°C to 25°C , with data taken at 5°C intervals. The deformation amplitude was $13 \mu\text{m}$, corresponding to a relative amplitude of 4×10^{-4} in the 3-cm long sample. The phase meter of this instrument resolves E^* into its elastic (E') and viscous (E'') components; $E^* = E' + iE''$. In each experiment, a sample ($30 \text{ mm} \times 6 \text{ mm} \times 2 \text{ mm}$) was mounted in an environmental chamber consisting of a hollow cooled copper block filled with pre-cooled dry nitrogen vapor. A sample of tissue about $30 \text{ mm} \times 5 \text{ mm} \times 1 \text{ mm}$ was clamped in place through a copper tab at each end, cemented to the sample with cyanoacrylate glue. In order to make the thermal contraction of the sample isometric during cooling down, one clamp was gradually moved inward, keeping the force on the sample small. (This was accomplished automatically with a computer-controlled stepping motor.) The chamber was then allowed to warm up to room temperature slowly, while the dynamic modulus was being measured.

Each viscoelasticity experiment was repeated sequentially with three or four separate specimens that had been treated simultaneously in the same solutions. The specimens had been excised from the butt of the same animal and were oriented with their long directions perpendicular to the animal's backbone. Bias in the orientation of the fibers in this direction was thereby held as constant as possible among specimens. Because measurement of the modulus of elasticity of our specimens required that they be held flat in space, a small pre-stress had to be applied. This could be kept small, about 0.05 N, with corresponding small strain in these impregnated materials. The modulus components were averaged at each temperature to give average curves of E' and E'' versus temperature. The scatter, which was ordinarily 10 to 20 percent, is shown in the figures as error bars representing the standard error of the mean of E' or E'' at each temperature. To determine whether a treatment changed the mechanical behavior, we observed whether the dynamic mechanical property curves, E' and E'' versus temperature, were vertically displaced beyond the extents of these error bars. The master curves of Fig. 5 were obtained by temperature-shifting the averages, using the procedure described by Ferry [1].

Results

Figure 1 shows the viscosity of 70 percent ethylene glycol solutions of chondroitin sulfate at low temperature. The concentration of the 1 percent glycosaminoglycan solution is about 6 percent of that we estimate for the interfibrillar gel in skin, given the available volume and the GAG content. Even small amounts of GAG, above 0.1 percent, appeared to aid super-

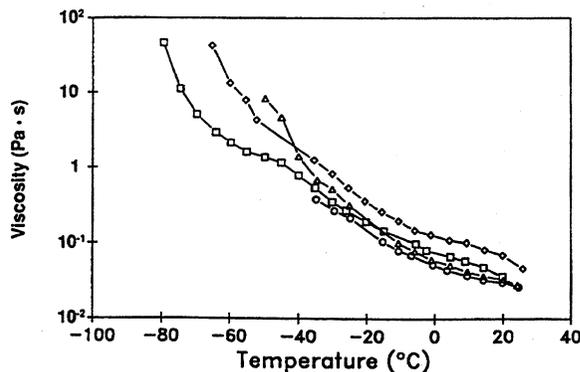


Fig. 1 Static viscosity of solutions of chondroitin sulfate in 70 percent aqueous ethylene glycol. [circles] 0 percent, [squares] 0.1 percent, [triangles] 0.25 percent, [diamonds] 1 percent (w/w).

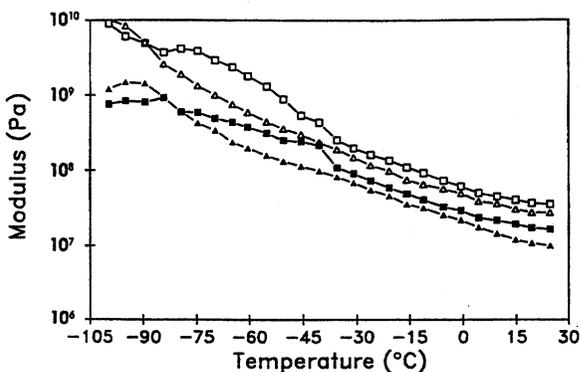
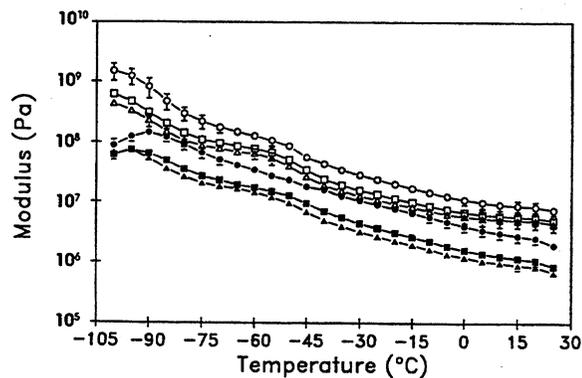


Fig. 2 Dynamic mechanical properties, measured at 110 Hz, of two specimens of calfskin impregnated with aqueous ethylene glycol at different concentrations: [square] 50 percent, [triangle] 70 percent. Open symbols: E' ; filled symbols, E'' .

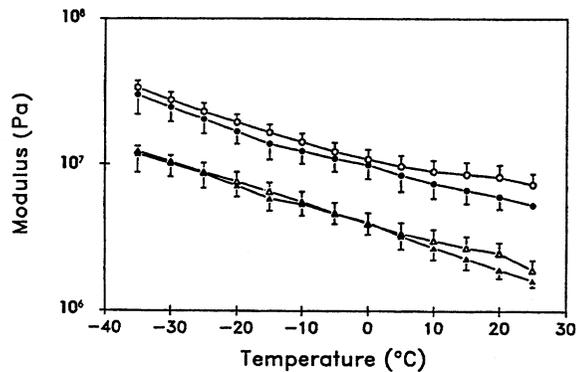
cooling. At 210 K the viscosity of a 1 percent GAG solution is about 14 Pa·s, about two orders of magnitude less than a typical value of the dynamic viscosity $E''/3\omega$ for calfskin at the lowest temperature (vide infra), where ω is the angular frequency of the measurement [1]. The two viscosities are not necessarily expected to agree, since $E''/3\omega$ is the viscosity of the composite system, not only of the interfibrillar gel.

Neither modulus, E' nor E'' , of impregnated calfskin changes significantly when the concentration of ethylene glycol is decreased from 70 to 50 percent (Fig. 2). The behavior of skin impregnated with lower concentrations is more difficult to compare, however, because in such materials the larger pre-stress that must be applied (see Methods) when using the Rheovibron elongates the sample and orients the fibers. The elastic modulus of skin is very sensitive to the fiber orientation [11], a problem which is somewhat obviated when the material is impregnated with an organic liquid, which makes the fibers stiffer and so less sensitive to handling. Figure 3 shows the dynamic mechanical properties of untreated calfskin. Each curve is the mean of three experiments with scatter commonly 10 to 20 percent. The elastic modulus is about 10 times the loss modulus at all temperatures and frequencies. Both moduli decrease steadily as the temperature is raised from -90°C , and they increase slightly with frequency, as expected for polymers. An unexpected maximum between -60°C and -50°C , which appears in all our experiments with 70 percent ethylene glycol, corresponds to the recrystallization temperature of the solvent. As seen in Fig. 3(b), salt extraction removing unidentified high-molecular weight material has no effect on the dynamic mechanical properties of calfskin, determined over a wide range of temperature and frequency.

Student's t -test applied to the means from the treated and untreated samples ($n = 3$) shows that only between -50°C



(a)



(b)

Fig. 3 Effect of extraction with sodium chloride on dynamic mechanical properties of calfskin in 70 percent ethylene glycol determined at different frequencies. Fig. 3(a) Effect of frequency on untreated skin: [circle] 110 Hz, [square] 11 Hz, [triangle] 3.5 Hz. Open symbols, E' ; filled symbols, E'' . b) Effect of extraction: open symbols, untreated; filled symbols, extracted. [circle] E' , [triangle] E'' . 110-Hz data at temperatures above the recrystallization range, plotted on an expanded scale, are presented in Fig. 3(b) to emphasize the close agreement between treated and control samples, $n = 3$.

and -60°C , where the solvent is recrystallizing, are the data for the treated and untreated tissues significantly different ($p < 0.05$). Of course one does not expect reproducibility over this interval.

Although calfskin has a complicated composite structure, we have considered the possibility that the relaxation times of the molecules of its components effectively all have the same temperature dependence. The material would behave in this way, for example, if the relaxation times of the collagen fibrils were far removed from those of the interfibrillar gel, even if their temperature dependencies were different. Here, each group of relaxation times would have its common temperature dependence, but only one group could match the experimental timescale. This is a likely condition when the two components of the system are as different from each other as gel and fiber. Theories for mechanical relaxation of gels predict the common temperature dependence of relaxation times [1]. The relaxation times of fibers themselves are expected to be very short. In such a circumstance the time and temperature dependences of the complex moduli would be interconvertible, and the moduli could be time-temperature shifted, following principles that have become well established in polymer physics [1]. Accordingly, the two moduli at each temperature were plotted against frequency and then shifted along the frequency axis by a factor of a , to construct two master relaxation curves. The reference temperature was chosen to be the center of the range, -40°C , and data at other temperatures were shifted relative to data at -40°C . The amounts of the shifts are plotted in Fig. 4 for E' and E'' . A test of the reliability of the assumptions underlying this treatment of data is the agreement between the shifts for the two moduli. As seen in Fig. 4, the agreement is

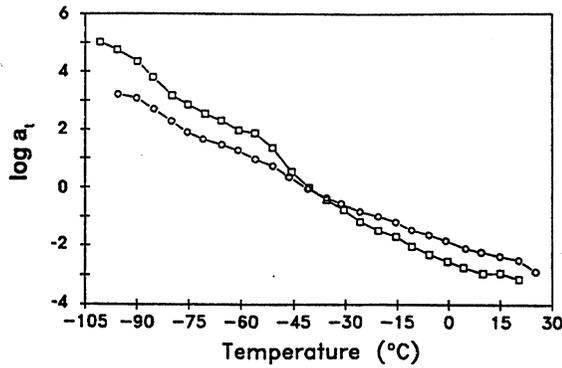


Fig. 4 Time-temperature shift factors for dynamic modulus of calfskin. [squares] E' ; [circles] E'' .

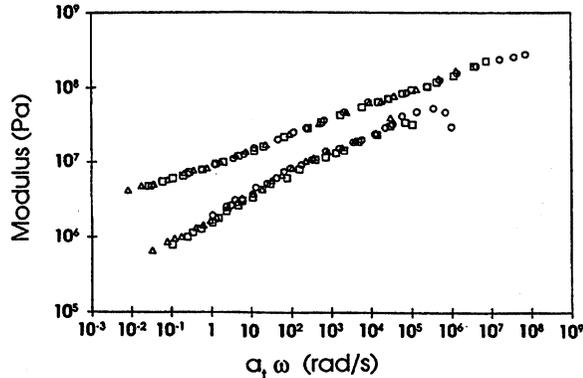


Fig. 5 Master curves of the components of the dynamic modulus of untreated calf skin, determined over eight decades of reduced angular frequency, constructed by horizontally shifting data from Fig. 3(a) according to the time-temperature superposition principle, using the shift factors of Fig. 4. [circle] 110 Hz; [square] 11 Hz; [triangle] 3.5 Hz; upper curve, E' ; lower curve, E'' .

only fair, considering that the vertical axis is logarithmic. The result of the treatment is shown in Fig. 5, which, subject to the above assumption, represents the behavior over a time scale of ten decades of frequency.

There is no discernible effect on the low-deformation mechanical properties after removal of over 99.7 percent of the hyaluronic acid by treatment with hyaluronidase (Fig. 6). Only the 110-Hz data are shown in Fig. 6; data at the other two frequencies are similar, with the usual slight shift of the curve to higher temperatures (q.v. Fig. 3). Student's t -test applied to the means from the treated and untreated samples ($n = 3$) shows that only over the freezing interval of the solvent, -50°C to -60°C , and below -75°C , are the moduli of the treated and untreated tissues significantly different ($p < 0.05$). The loss of hyaluronan seems to lower both moduli at very low temperature, probably by suppressing very rapid motions or by changing the properties of the glass-forming solvent.

Figure 7 shows that treatment with chondroitinase ABC, which effectively removed dermatan sulfate, lowered both moduli, but only at temperatures above -50°C . The changes seem small on the five-decade logarithmic modulus scale, but actually amount to as much as a factor of two. Student's t -test applied to the means from the treated and untreated samples ($n = 4$) shows that above -45°C the data for the treated and untreated tissues are significantly different ($p < 0.05$).

The mechanically active components of the system resist deformation only as long as it is more rapid than their relaxations. Faster relaxations therefore play no role in defining the moduli. The result of removal of GAG with chondroitinase ABC—the moduli becoming smaller under high-temperature, short-time-scale conditions—can be interpreted as a selective effect on the more slowly relaxing components of the system.

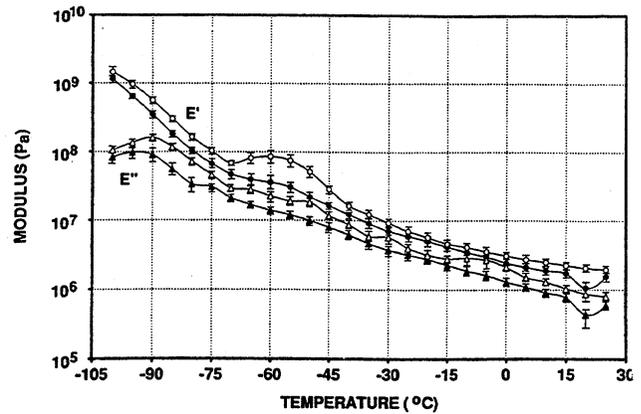


Fig. 6 Effect of treatment with hyaluronidase on the 110-Hz dynamic mechanical properties of calfskin. Filled symbols, treated; open symbols, control. [circle] E' ; [triangle] E'' . $n = 3$.

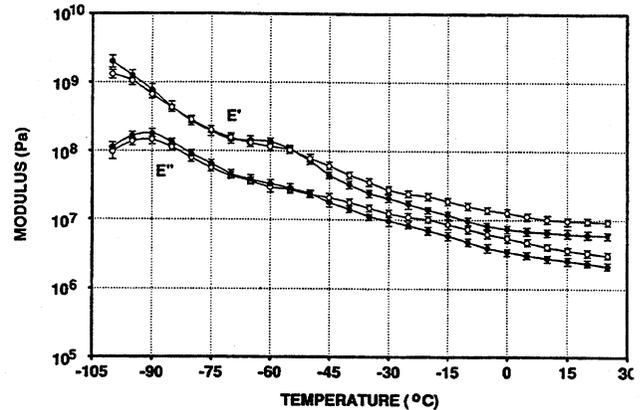


Fig. 7 Effect of chondroitinase ABC on the 110-Hz dynamic mechanical properties of calfskin. Filled symbols, treated ($n = 3$); open symbols, control ($n = 4$). Upper curves, E' ; lower curves, E'' .

Such components are expected to be macromolecular, including not only GAG but also proteins.

We then examined the effects at longer relaxation times due to removal of noncollagenous, interfibrillar protein by treatment with trypsin. We reduced the amount of ethylene glycol to bring the slowly-relaxing components into our experimental range and diverted our attention to the temperature region above -45°C . Figure 8(a) shows that with less ethylene glycol the moduli again are diminished above -45°C by a factor that approaches two at room temperature. Student's t -test applied to the means from the treated and untreated samples ($n = 3$) shows that above -45°C the data for the treated and untreated tissues are significantly different ($p < 0.05$).

The results of skin in 70 percent ethylene glycol treated with trypsin (Fig. 8(b)) also resembled those treated with chondroitinase ABC. These moduli were different at ($p < 0.05$) down to -75°C , according to Student's t -tests at each temperature.

Discussion

Temperature Dependence of E^* . The interpretation of the components of the complex modulus E^* in a structure comprising fibers and a matrix, both viscoelastic, is not straightforward. The temperature dependence of the viscosity of neat ethylene glycol and with chondroitin sulfate as solute show that the E'' at low temperature is not just an effect of the increasing viscosity of the cooling solvent. The viscosities of ethylene glycol solutions increased by over three orders of magnitude, while that of the tissue, calculated by the formula viscosity = $E''/3\omega$, increased by less than two. The changes

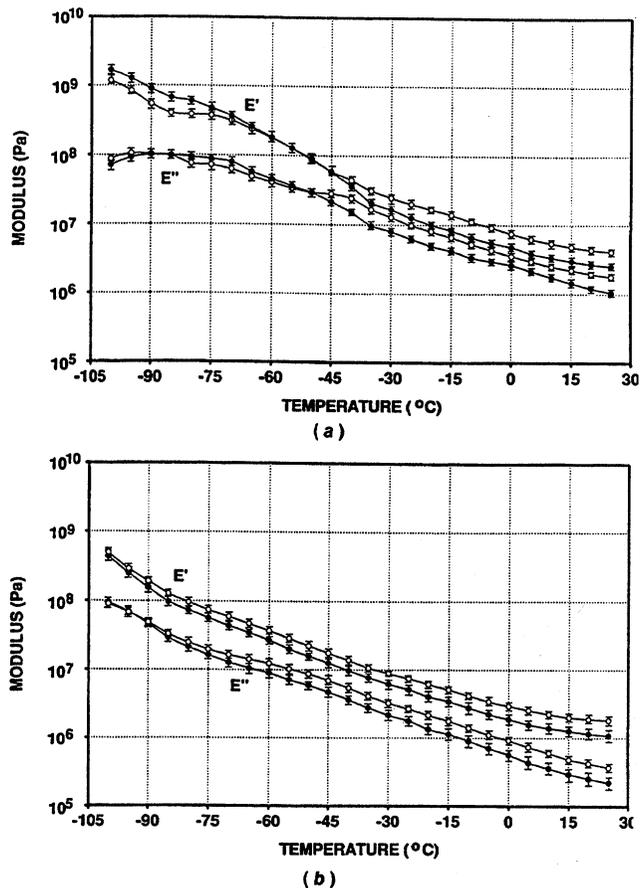


Fig. 8 Effect of trypsin on the 11-Hz dynamic mechanical properties of calfskin. Filled symbols, treated; open symbols, control. Upper curves, E' ; lower curves, E'' . Fig. 8(a) Impregnation solvent was 50% ethylene glycol. Fig. 8(b) Impregnation solvent was 70% ethylene glycol. $n = 3$.

were due to the interaction of the fibers and matrix and led to a much higher value of viscosity than that of the matrix alone. These interactions are affected by the viscosity of the matrix gel, however, and depend on the mechanical relaxation-time spectrum of the macromolecules dissolved in it.

Exploring the relaxation-time spectrum is best done with measurements made over a wide range of time scales. This is not possible in mechanical measurements with a single apparatus. Instead, the relaxation times themselves are usually shifted into the accessible range by working at cryogenic temperatures. For biological tissue this obviously requires replacing water by a low-melting solvent that mimics it in ways essential to preservation of molecular configurations—a solvent that is hydromimetic at low temperatures. The GAG portion of the gel, which might be affected by such a solvent, is a polyelectrolyte; from the chemical composition one can estimate the fixed charge density to be about 0.2 meq/cm^3 gel. (GAG, 1 percent of the hide by mass [12] and concentrated into about 0.6 of its volume, is then 1.7 percent of the interfibrillar gel. Dry, it contains 10 meq of fixed charges per gram, giving 0.2 meq/g gel). The configurations and the states of aggregation of polyelectrolyte molecules depend on the ionic strength and the dielectric constant of the fluid medium in the matrix. Further, it has been shown that the configurations of proteins remain native in aqueous ethylene glycol mixtures as long as the temperature is lowered enough to hold the dielectric constant at 80. Thus many enzymatic activities are preserved (although thermally slowed), and details of structure as determined by X-ray diffraction are unaltered except for more restricted movements of side chains [2]. Therefore it seemed that aqueous ethylene glycol could serve as the low-temperature hydromimetic solvent needed for extended studies of viscoelasticity.

Notwithstanding the reason for our choice of low-temperature solvent—that it would maintain high dielectric constant at low temperature—the most interesting part of the data is in the higher temperature region, where the dielectric constant is lower than 80. Thus, although at -50°C the dielectric constant of 70 percent ethylene glycol is 84.5, it is only 56.9 at 20°C [2]. All theories of polyelectrolytes, which include GAG, require that the macromolecules be folded up when the dielectric constant of the solvent is small. Nevertheless the large viscosities of chondroitin sulfate-ethylene glycol solutions shown in Fig. 1 indicate that at the concentrations of organic solvent used here, the GAG molecules are in extended conformations even at room temperature. The temperature dependence of E^* is thus most likely not due to the changing dielectric constant. The E^* of the calfskin remained almost the same (Fig. 2) when the dielectric constant of the solvent decreased from 89.3 in 50 percent ethylene glycol at -50°C to 84.5 in 70 percent ethylene glycol, and at 20°C from 64.5 to 56.9 [2]. Although the GAG molecules are extended, the interfibrillar proteins might be collapsed into more compact masses. Certainly, replacement of water by the solvent causes the storage modulus of the collagen fibrils to rise [13]. We have also found that evaporation of water from synthetically crosslinked collagen fibrils causes the loss modulus of the material to fall [14]. We therefore attribute the rise of the loss modulus in the presence of solvent to effects on the interfibrillar matrix.

Effects of Removal of Noncollagenous Macromolecules.

Using a theory often applied successfully to polymer gels [1] (q.v. Fig. 5), we showed that treatment with salt or hyaluronidase, which remove soluble biopolymers from the interfibrillar matrix, has no detectable effect (Fig. 3, Fig. 6) on the viscoelasticity of calfskin measured over a very wide time scale, from the equivalent of submicrosecond times to 102 s at -40°C (The master curves are not shown here). The lack of effect of salt extraction might have been expected, since it removes only about one third of the noncollagenous macromolecules in leathermaking processes [12]. Hyaluronan appears not to affect the mechanical properties, perhaps because its molecules are not attached to the fibrils. Removal of the proteins or polysaccharides known to be attached to the fibrils, however, reduced the two components of modulus by half, measured near room temperature. The difference would most obviously be due to the attachment of these biopolymers to collagen fibrils [15], causing them to be deformed more or to shear the solvent more rapidly and so to increase the external elastic and viscous forces on the fibrils. It is interesting to observe that the effect of the enzyme vanished at low temperatures (equivalent to short time scales). Under conditions where the solvent is very viscous, the fibrils have little time to reorient with respect to each other, the GAG relaxing but little and forcing the fibrils to move in concert. At longer times, on the other hand, the fibrils have time to move with respect to each other. This additional freedom should be sensitive to the composition of the matrix gel and, in agreement, was found to increase when the macromolecules of the gel were removed. Of the two enzymes used, trypsin would be expected to release both proteins and DSPG, because the protein cores, not the GAG chains, are believed to bind DSPG to collagen fibrils [15].

A fundamental challenge in bioengineering is the uniqueness of the structure and mechanical properties often attributed to tissue. Skin, for example, comprises a hierarchy of sizes of collagenous fibers, connected by a fine reticulum of very fine fibrous structures composed, as presently known, of type VI collagen-based microfibrils [16], fibrillin-based microfibrils [17], and an attached molecular network of the dermatan-chondroitin-sulfate proteoglycan, decorin (small proteoglycan II) [18]. Elastic fibers, on the other hand, are not a significant component of bovine reticular dermis. A mechanically match-

ing implant would have to have a much simpler construction, but with the same mechanical and physiological properties. The importance of these substructures should then be established in order to define goals in the design of organ implants, especially those comprising large amounts of connective tissue such as skin, tendons, and bones. We have found here that the part of the interfibrillar matrix that is important is attached to the fibrils: decorin and possibly protein-based microfibrils. When these were removed, both the elastic modulus (controlling the immediate mechanical response) and the loss modulus (controlling the time-dependent response) decreased by almost half. Removal of hyaluronan, which seems to be free in the interfibrillar solution, had no effect on the dynamic mechanical properties at any temperature. If a fiber-reinforced composite is to be designed that matches this behavior, both its fibers and its matrix must be viscoelastic. The constants of such a composite could be determined from data such as ours from an analysis like that of Takayanagi [19], who represented the experimental composite being investigated as a series-parallel arrangement of two blocks of viscoelastic materials, for which the components of the two E^* 's could be fit to the data. Exact values of the constants for skin, however, may not be taken from the present data because of the effect of the organic solvent on the properties of the fibers and the short times of the responses that were explored.

An issue might be raised about the specificities of the three enzymes used: whether they or their impurities might attack the collagen (types I, III, V) fibrils themselves. The chondroitinase ABC has no detectable proteolytic or collagenolytic activity. The trypsin, however, might have attacked the collagen of the fibrils at locally unfolded sites, but necessarily at our temperature of treatment to an extent much less than that to which it attacked the interfibrillar proteins, including the protein parts of the proteoglycans, which bind these complex macromolecules to the fibrils [16]. Both the chondroitinase and the trypsin could have solubilized the GAG's. We found here however that the effect of the trypsin on the components of the complex modulus was no greater than that of the chondroitinase ABC. It would then seem highly unlikely that the effect of the mechanical properties of calfskin from the trypsin we used, which was of the highest purity available, was due to its promotion of the degradation of fibrillar collagens. (The point could not be proven by analysis for traces of fragments containing hydroxyproline, an amino acid that is characteristic of collagen, because it would also have been liberated by trypsin attacking slightly denatured collagen, or collagen precursors and fragments that exist in natural calfskin, which do not contribute to the elastic properties of the fibrils.) It is most probable that the similar effects of the two enzymes on E' and E'' are due to their common degradation of fibril-bound decorin and not to effects on collagen of types I, III, V, or VI, or hyaluronan.

Relevance to Bioengineering. This work addresses problems in the biomechanics of several flat, soft organs that arise in surgery such as incisions into skin or pericardium, wound healing, and transplantation. The behavior studied is also of concern in the design of artificial skin and prostheses. Also, the engineer wants to know the underlying mechanisms of the systems he works with in order to effect his control. Dermis was selected here as the connective tissue for study because we decided that its loose, web-like structure would serve as a more general model than tendon, with highly oriented fibers, or osmosis-supported cartilage. Calfskin was suited for the source of the dermis because of its ease of dissection from the epidermis to a thickness that was compatible with our apparatus. As a step toward our long-range objectives of relating the properties and functions of connective tissues to their structures, we evaluated the effects of selected macromolecular constituents of the interfibrillary matrix on the elastic and

viscous components of the modulus of calfskin. The general method was to vary the structure by selective removal of bound or soluble GAG or protein. These categories were broad because it was not known in advance how large would be the effects of removing even such large classes of macromolecules on the time-dependent mechanical responses. Future studies can focus on more specific proteins and proteoglycans.

Conclusions

Removal of free macromolecules, including hyaluronan, by salt extraction or treatment with hyaluronidase does not affect the viscoelasticity of bovine reticular dermis.

The viscoelasticity is sensitive to the presence of macromolecules and microfibrils bound to the 100-nm collagen fibrils.

Valid mechanical data on soft tissue can be obtained at low temperatures by impregnating it with hydromimetic solvents that do not perturb electrostatic conditions at those temperatures. Such a solvent is 70 percent ethylene glycol.

References

- 1 Ferry, J. D., *Viscoelastic Properties of Polymers*, 3rd ed., Wiley, New York, 1980, pp. 266-312, 487-544; Bird, R. B., Curtiss, C. F., Armstrong, R. C., and Hassager, O., *Dynamics of Polymer Liquids*, 2nd ed., Wiley, New York, 1987.
- 2 Douzou, P., and Petsko, G. A., "Proteins at Work," *Adv. Prot. Chem.*, Vol. 36, 1984, pp. 246-362; F. Travers, P. Douzou, C. Pederson, and F. Gun-salus, *Biochimie*, Vol. 57, 1975, pp. 43.
- 3 Partington, F. R., and Wood, G. C., "The Role of Non-collagenous Components in the Mechanical Behavior of Tendon Fibres," *Biochem. Biophys. Acta*, Vol. 69, 1963, pp. 485-495.
- 4 Dale, W. C., "A Composite Materials Analysis of the Structure, Mechanical Properties and Aging of Collagenous Tissue," Ph.D. thesis, Case West-ern Reserve University, 1974.
- 5 Oxlund, H., and Andreassen, T. T., "The Roles of Hyaluronic Acid, Collagen and Elastin in the Mechanical Properties of Connective Tissues," *J. Anat.*, Vol. 131, 1980, pp. 611-620.
- 6 Viidik, A., Danielsen, C. C., and Oxlund, H., "Fundamental and Phenomenological Models, Structure and Mechanical Properties of Collagen, Elastin and Glycosaminoglycan Complexes," *Biorheology*, Vol. 19, 1982, pp. 437-451.
- 7 Roden, L., in Colowich, S. P., and Kaplan, N. O., eds., *Methods in Enzymology*, Vol. 28, 1972, pp. 77.
- 8 Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S., "Purification and Properties of Bacterial Chondroitinases and Chondrosulfatases," *J. Biol. Chem.*, Vol. 243, 1968, pp. 1523-1542.
- 9 Bitter, T., and Muir, H. M., "A Modified Uronic Acid Carbazole Re-action," *Anal. Biochem.*, Vol. 4, 1962, pp. 330-334.
- 10 Woessner, Jr., J. F., "The Determination of Hydroxyproline in Tissue and Protein Samples Containing Small Proportions of This Imine Acid," *Arch. Biochem. Biophys.*, Vol. 93, 1961, pp. 440-447.
- 11 Kronick, P. L., "Analysis of the Effects of pH and Tensile Deformation on the Small-Deformation Modulus of Calf Skin," *Conn. Tissue Res.*, Vol. 18, 1988, pp. 95-106.
- 12 Bienkiewicz, K., "Physical Chemistry of Leathermaking," Robert E. Krieger, Malabar, FL, 1983, p. 297.
- 13 Kronick, P. L., and Buechler, P. B., "Fiber Orientation and Small De-formation Modulus of Stretched, Partially-Dried Hide," *J. Am. Leather Chem-ists Assn.*, Vol. 83, 1988, pp. 115-124.
- 14 Kronick, P. L., Buechler, P., Scholnick, F., and Artymyshyn, B., "Dy-namic Mechanical Properties of Polymer-Leather Composites," *J. Appl. Polym. Sci.*, Vol. 30, 1985, pp. 3095-3006.
- 15 Scott, P. G., Winterbottom, N., Dodd, C. M., Edwards, E., and Pearson, C. H., "A Role for S-S Bridges in the Protein Core in the Interaction of Proteodermatan Sulphate and Collagen," *Biochem. Biophys. Res. Comm.*, Vol. 138, 1986, pp. 1348-1354.
- 16 Keene, D. R., Engvall, E., and Glanville, R. W., "Ultrastructure of Type VI Collagen in Human Skin and Cartilage Suggests an Anchoring Function for This Filamentous Network," *J. Cell Biol.*, Vol. 107, 1988, pp. 1995-2006.
- 17 Sakai, L. Y., Keene, D. R., and Engvall, E., "Fibrillin, A New 350-kD Glycoprotein, Is A Component of Extracellular Fibrils," *J. Cell Biol.*, Vol. 103, 1986, pp. 2499-2509.
- 18 Fleischmajer, R., Fisher, L. U., MacDonald, E. D., Jacobs, L., Perlsh, J. S., and Termine, J. D., "Decorin Interacts with Fibrillar Collagen of Em-bryonic and Adult Human Skin," *J. Structural Biol.*, Vol. 106, 1991, pp. 82-90.
- 19 Takayanagi, M., Uemura, S., and Minimi, S., "Application of Equivalent Model Method to Dynamic Rheo-Optical Properties of Crystalline Polymer," *J. Polym. Sci.*, C5: 113 1964.

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.