AN INVESTIGATION OF THE BOUND WATER IN TENDON BY DIELECTRIC MEASUREMENT

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ABSTRACT The dielectric constant of natural tendon in the frequency range of 200 Hz to 100 kHz has been determined as a function of temperature (300–450°K) for water concentrations ranging from about 6 to 16% by weight. The results compare well with the approach taken by Haggis and his coworkers. Based on the assumption that at low concentration of water the probability of water-water bonding is small and hence may be disregarded, a structure for water in the collagen matrix is proposed in which the water is either bonded in one of four possible states to the polar groups of the polypeptide chains, or is unbound. In determining the distribution of the water among these states an approach similar to that of Haggis and his coworkers, in conjunction with the order-disorder theory of Bragg and Williams, is used. The number of water molecules per unit volume is then determined experimentally by relating it to weight loss as a function of temperature, as determined by thermogravimetric analysis. The dispersion which is normally found in dipolar substances has not been found for tendon. A maximum in the value of the dielectric constant is observed to occur between 25 and 80°C, the temperature depending upon the heating rate.

INTRODUCTION

In recent years intensive efforts have been made to understand the nature of fibrous collagen in connective tissue and bone by studying the electrical properties of these materials. It has been shown that this collagen is piezoelectric and there are indications that all materials containing collagen are piezoelectric (1). As with other piezoelectric substances, these materials can act as transducers (e.g., tendon has been used as an electromechanical pickup for a phonograph [2]). The role which this transducer properly plays in vivo is not fully understood at present although it is believed to be related to the growth and remodeling of tissue and possibly to tactual processes (1).

Studies of the dielectric properties of bone have been carried out by Freeman (3) and by Marino et al. (4). Freeman emphasized the importance of bound water in

the bone matrix and the effect of diffusion on the dielectric properties of the system. Marino et al. determined the amount of bound water present in bone. Their results indicate that the amount of water necessary to occupy the primary absorption sites in bone, i.e. the bound water of the system, ranges from 3.7 to 4.8% by weight, depending upon the density of the bone. They further observed that the bound water does not make any dipolar reorientation contribution to the dielectric constant, but rather is like a nonpolar system, contributing only to the temperature-independent electronic and molecular polarizability.

The dielectric behavior of rat tail tendon collagen in dilute acetic acid has been reported by Hauss et al. (5). They found that after collagen had been heated to a temperature between 60 and 100°C it showed a dielectric dispersion similar to debyian relaxation. The relaxation energy of this dispersion was not reported.

To date the dielectric properties of natural tendon have not been reported. Thus we have undertaken a series of measurements of the dielectric behavior of natural tendon as a function of temperature, with water content as a parameter. The study of this bound water in the collagen matrix is important both because of its relationship to the structure of collagen and its effect on the electrical properties of the system. Dielectric measurements were chosen to study the bound water because of their sensitivity to the amount of water present and to the distribution of this water among the possible states of the system. Natural bovine Achilles tendon has a collagen content which ranges from about 70 to 90% by weight.[1] Thus, to understand the role of water in tendon one must know the basic structure of collagen.

Collagen is a macromolecule made up of protein molecular residues organized in the form of a triple helix. This triple helix is stabilized by hydrogen bonding between amide groups (N—H) of one peptide chain and carbonyl group (C=O) of another chain. It is believed that these intramolecular hydrogen bonds are the mechanism responsible for the piezoelectric effect in collagen (1, 2).

Two models have been proposed for collagen, one by Rich and Crick (6) in 1961 and the other by Ramachandran and Kartha (7) in 1955, which was modified slightly in 1965 (8). The most important difference between these two models is the number of intramolecular hydrogen bonds which they have. Rich and Crick's model has one NH——OC hydrogen bond for every three residues of a chain whereas in Ramachandran's model there can be three hydrogen bonds for every three residues of a chain: two NH——OC bonds and one CH——O bond. Although the dispute over the number of intramolecular hydrogen bonds has not yet been resolved, a recent study of the enthalpy change as a function of water content in the helix-coil transition of tendon tends to favor the Rich-Crick model.[2]

A number of different structures for the water bound to the polypeptide chains of the collagen molecule have been proposed. Berendsen (9), using the Rich-Crick

[1] Maxur, A. J., and M. H. Shamos. Unpublished data.

[2] De Lisi, C., and M. H. Shamos. Manuscript submitted for publication.

model, estimated that for a given 100 amino acid residues there are 114 sites available at which water can be bound to the polar groups of the peptide chains. This would indicate that about 20% of the water in collagen can be bound to polar sites. He further discusses the possibility of the formation of water chains between rodlike protofibrillar crystalline structures. Both De Lisi and Shamos[2] and Poland and Scheraga (10), in their studies of the collagen-water system, have used an alternate structure in which it is assumed that the water initially exists unbound in the cavities of the collagen chains and then at the transition temperature, when the intramolecular hydrogen bonds (NH——OC) which stabilize the collagen molecule break cooperatively, this water becomes bound to the polar sites provided by the freed NH and CO groups.

In any event, there can be little doubt that there exist a large number of bound water molecules in the collagen matrix, even though the actual water concentration is low. Thus, we will propose a structure for water in collagen at the low concentrations involved in our dielectric measurements.

EXPERIMENTAL PROCEDURE AND RESULTS

Sample Preparation

Bovine achilles tendon from a cow that was from 4 to 5 yr old was obtained from the slaughterhouse immediately after the animal had been slaughtered. After the elastic sheath had been cut away under cold running water, the tendon was air-dried at room temperature for 2 days. The air-dried tendon was then glued to a plate and cut to the desired size with a diamond saw cooled either by cold running water or by alcohol. After the sample had been cut, it was again air-dried for a day.

The water content of the sample was then adjusted to the desired concentration. For concentrations below the room temperature moisture content, this was done by evacuation for a specified time that depended both on the size of the sample and the concentration of diluent to be achieved. The samples were aproximately 0.1 cm by 1 cm by 2 cm rectangular slabs. To obtain a sample thickness of 0.1 cm without causing heat denaturation of the sample, the samples were first cut to a thickness of 2 mm and then slowly sanded down until a thickness of 1 mm was obtained. After the desired size had been achieved, the sample was washed with alcohol and vacuum-dried overnight. Silver epoxy was then applied to both sides of the sample and allowed to dry for 12 hr. Wire electrodes were attached to both sides with the same epoxy.

Measurements

For the dielectric measurements a General Radio type 1615-A modified Shering bridge (General Radio Co., Concord, Mass.) was used in conjunction with a General Radio type 1311 audio oscillator and type 1232-A tuned amplifier and null detector. This assembly is capable of measuring either the series or parallel capacitance over the frequency range of 100 Hz to 100 kHz. The sensitivity of most of the measurements, which were of the three-terminal type, was about ± 0.02 pF.

A schematic diagram of the temperature bath that was used is shown in Fig. 1. The temperature was controlled by a Fisher proprotional temperature controller (Fisher Scientific Co., Pittsburgh, Pa.). The glass sample holder was immersed in the heating bath which was

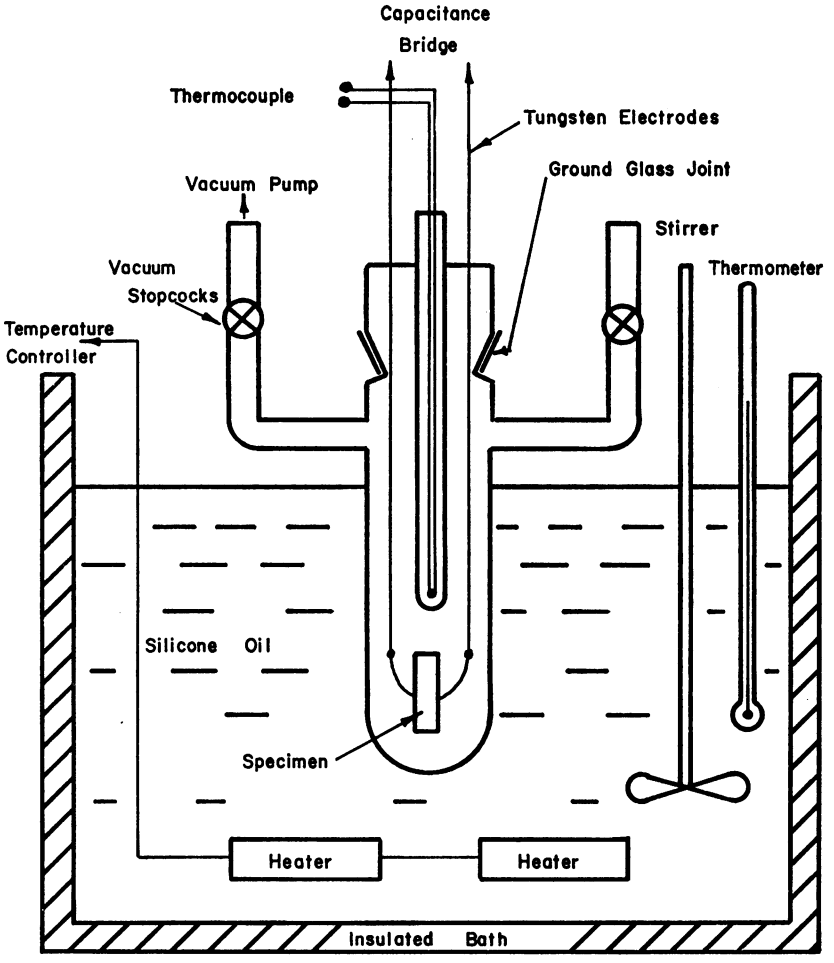

FIGURE 1 Schematic of oil bath.

filled with silicone oil. The bath contained a stirrer, electric heater, thermometer, and thermocouple.

Two types of measurements were made: equilibrium measurements in which the sample was heated to a series of increasing temperatures, waiting sufficiently long (from 24 to 36 hr) at each temperature for the sample to reach an equilibrium value of capacitance, and continuous measurements in which the sample was heated continuously at the rate of approximately 1°C/min from room temperature to the shrinkage temperature of the sample, the capacitance being measured at a number of points during this process.

The range of temperature variation in dielectric measurements is limited by distortion of the sample as the result of shrinkage, the shrinkage temperature in turn being determined by the content of diluent in the sample. Thus our dielectric measurements were carried out on samples that contained less than 16% water by weight.

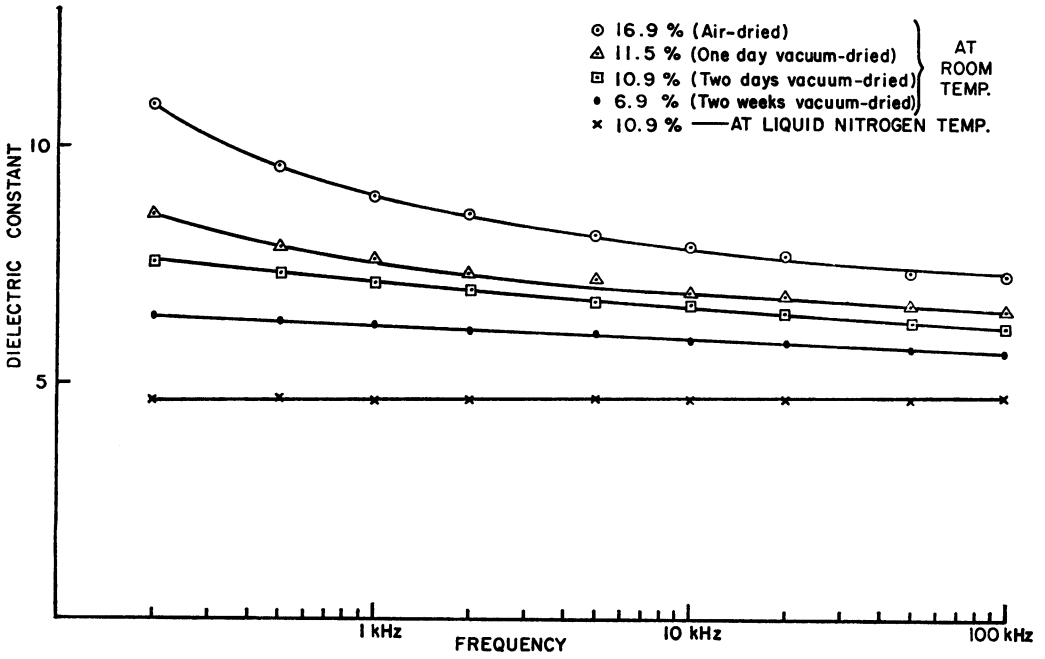

FIGURE 2 *a* Frequency dependence of the dielectric constant of tendon with water content as a parameter.

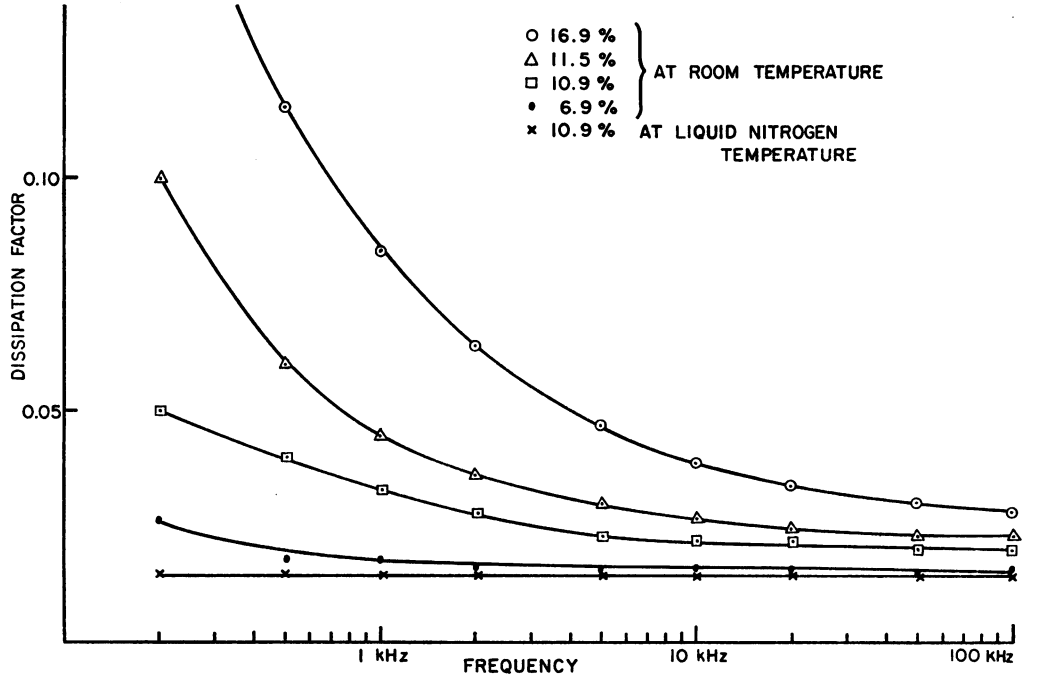

FIGURE 2 *b* Frequency dependence of the dissipation factor of tendon with water content as a parameter.

Results

The typical frequency dependence of the dielectric constant as a function of frequency is shown in Fig. 2 *a*. As can be seen, the variation in the dielectric constant as a function of frequency is sensitive to the water content of the sample, the evacuated sample (6.9–11.5% of water by weight depending on the length of evacuation) showing a smaller change in slope than the air-dried samples (16.9% by weight). The dissipation factors for the corresponding samples are given in Fig. 2 *b*. It is seen that the dissipation factor as a function of frequency is more sensitive to water content than the dielectric constant. For all samples the dielectric constant and the dissipation factor are found to decrease continuously as a function of frequency. No dispersion is observed in the frequency range of 200 Hz to 100 kHz.

The temperature dependence of the dielectric constant was found to vary widely with the rate of heating, which affects the number of water molecules present at the time of measurement, as is shown in Fig. 3 *a*. Curves *a* show the result of a continuous measurement (about 1°C/min of rate of heating) of the sample containing 10.9% water, curves *b* the result of a continuous measurement of the sample con-

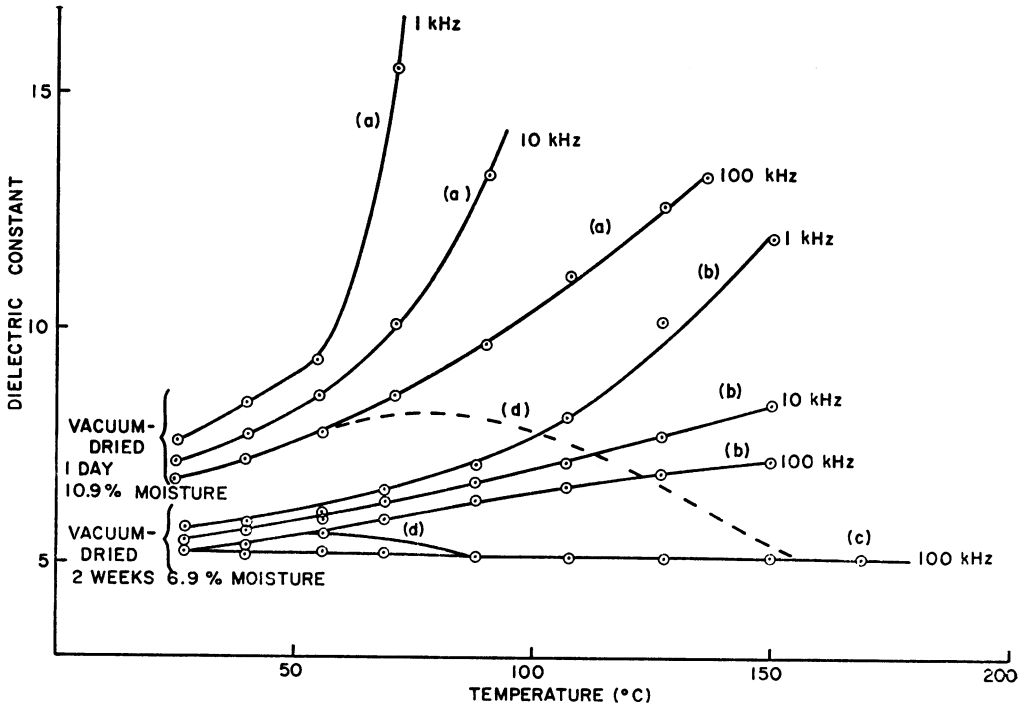

FIGURE 3 *a* Temperature dependence of the dielectric constant of tendon with water content and frequency as parameters: curves *a* and *b* are for continuous heating (heating rate 1°C/min); curve *c*, equilibrium measurement (after 24 hr); and curve *d*, intermediate states (3 hr between measurements). Curves *b*, *c*, and *d*, less than 30 μ pressure.

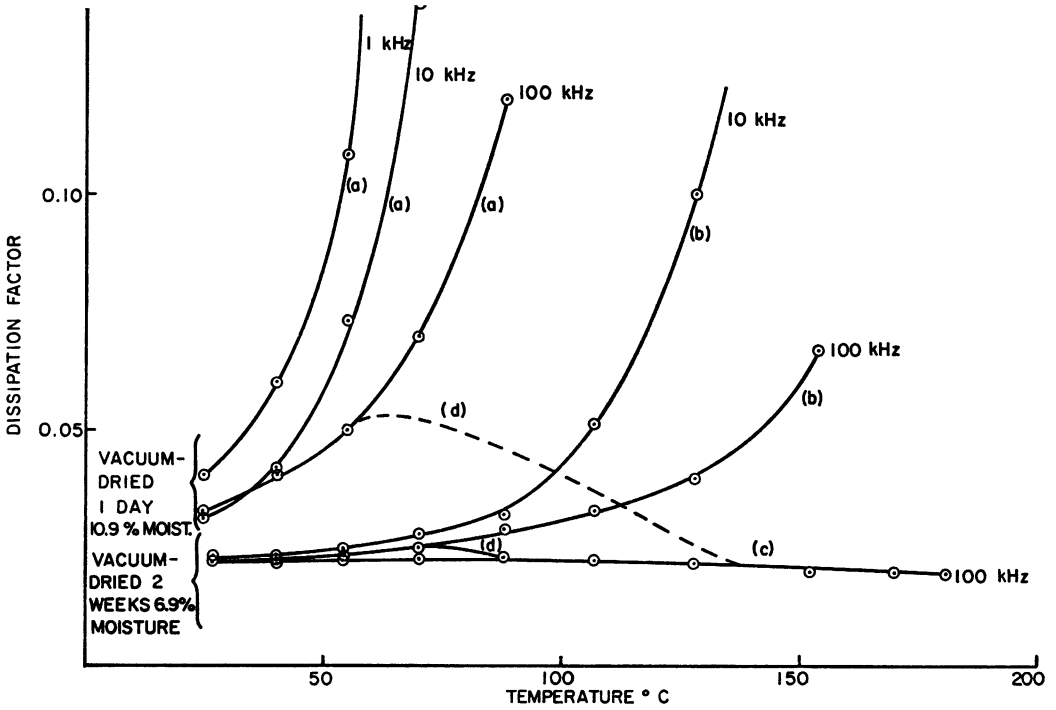

FIGURE 3 *b* Temperature dependence of the dissipation factor of tendon with water content and frequency as parameters. Conditions were same as in Fig. 3 *a*.

taining 6.9% water, curve *c* of an equilibrium measurement (about 24 hr for each measurement), and curve *d* at a heating rate of about 3 hr per measurement. The temperature at which the peak in curve *d* occurs depends on the rate of heating, increasing as the heating rate increases. The dissipation factor is found to increase more rapidly as a function of temperature at greater heating rates, as shown in Fig. 3 *b*.

The dielectric constant and dissipation factor as a function of the hydration, with frequency as a parameter, are shown in Figs. 4 *a* and 4 *b* respectively. These figures indicate that as the hydration increases the dielectric constant and dissipation factor increase.

Since the dielectric constant is strongly dependent upon hydration, we obtained the hydration by measuring the dielectric constant as a function of temperature. Fig. 5 shows the weight change as a function of temperature with hydration as a parameter, the results having been obtained with the Thermogravimetric Analyzer (TGA).

The melting of collagen is observed in the dielectric measurements, since when the sample melts the dimensions of the sample change, which results in a change in the measured capacitance.

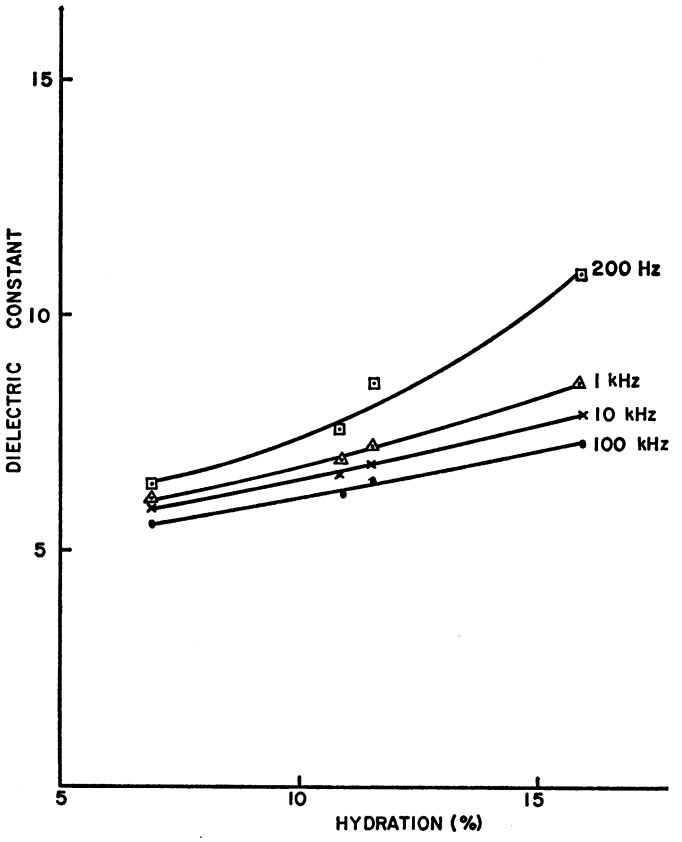

FIGURE 4 *a* Dielectric constant as a function of hydration with frequency as a parameter at room temperature.

DISCUSSION

In general, the dielectric constant stems from three sources: electronic, atomic (ionic), and molecular or dipolar polarizabilities. However, because in the range of our measurements the contributions of the electronic and atomic polarizabilities are essentially constant with respect to temperature, our main concern is with dipolar rotation. In most substances the dipolar contribution to the dielectric constant shows many interesting effects related to dispersion phenomena due to dipolar rotation (Debye type), and one can determine the dipole moment of the substance from the temperature variation of the dielectric constant. However, in our dielectric measurements no dispersion was observed. To understand this lack of normal dispersion, we must examine the possible dipolar sources in tendon.

There are three dipolar sources in tendon: the residues of the chains, the polar groups of the side chains, and the water. For any of these to contribute to dipolar

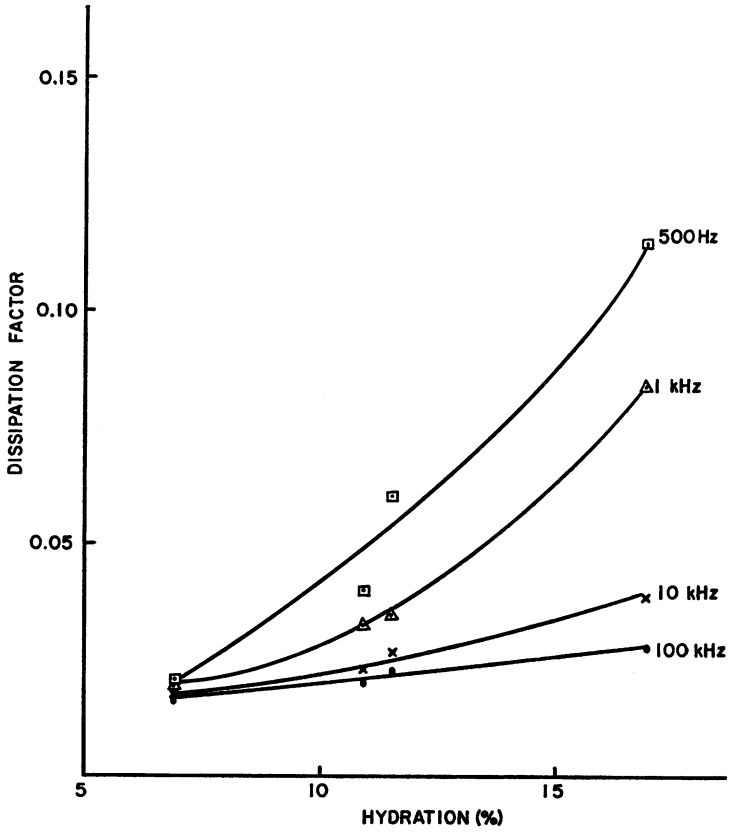

FIGURE 4 *b* Dissipation factor vs. hydration with frequency as a parameter at room temperature.

dispersion, they must be able to undergo cooperative rotation at a frequency which is characteristic of the substance and depends on the temperature.

We may safely disregard the possibility of rotation of the residues, since they are covalently bonded in a chain that is about 2800 A long. Each chain has many polar groups that are oriented almost perpendicular to the chain. Some investigators claim to have observed adsorption in denatured tendon which they attribute to the OH group of the hydroxyproline residue (11). However, since this polar group can readily form hydrogen bonds with one or more water molecules, as is shown in Fig. 6, it is unlikely that it is free to rotate. In general, since dispersion due to the polar groups of the peptide chain presupposes that they are free to rotate, any water that is bonded to them must break cooperatively at or before the temperature at which the dispersion is to be observed. Both the experimental evidence and the theoretical model which we will use to explain the bonding of water to these polar groups, a model which is consistent with the experimental observations, show that

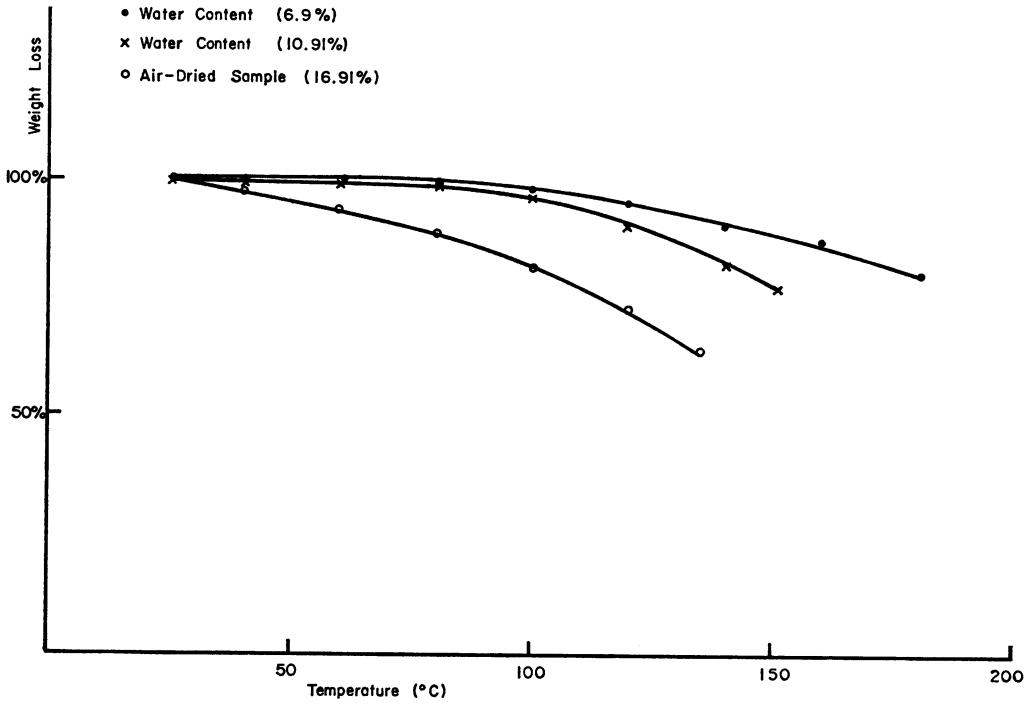

FIGURE 5 Weight change as a function of temperature with water content as a parameter. The rate of heating was 1°C/min.

the breaking of bonds between the polar groups and water is a continuous process rather than a cooperative one. Therefore, these polar groups cannot contribute to absorption in natural tendon. An argument exactly analogous to the one above shows that the bound water in tendon could not contribute to any absorption. Hence it is not surprising that absorption is not observed in natural tendon since its three possible sources have been eliminated.

Although the individual residues of the peptide chain in collagen and the water which is present are highly polar (12), these groups are bonded together in such a way (triple helix) that they do not show any dipolar characteristics. The unbound water will still be polar. The number of unbound water molecules will increase as the temperature increases, because the bonded water becomes unbonded.

The bound water in collagen may form either intra- or intermolecular hydrogen bonds or intrapeptide chain bonds between (CO), (NH), or (OH) groups of the polypeptide residues (13–15). There is the further possibility of water-water hydrogen bonds and consequently of the formation of water chains in the collagen matrix (13), but at low concentrations the probability of water-water bonding will be small and therefore will be disregarded in our treatment.

There are five possible states for water in collagen, one unbound and four bound

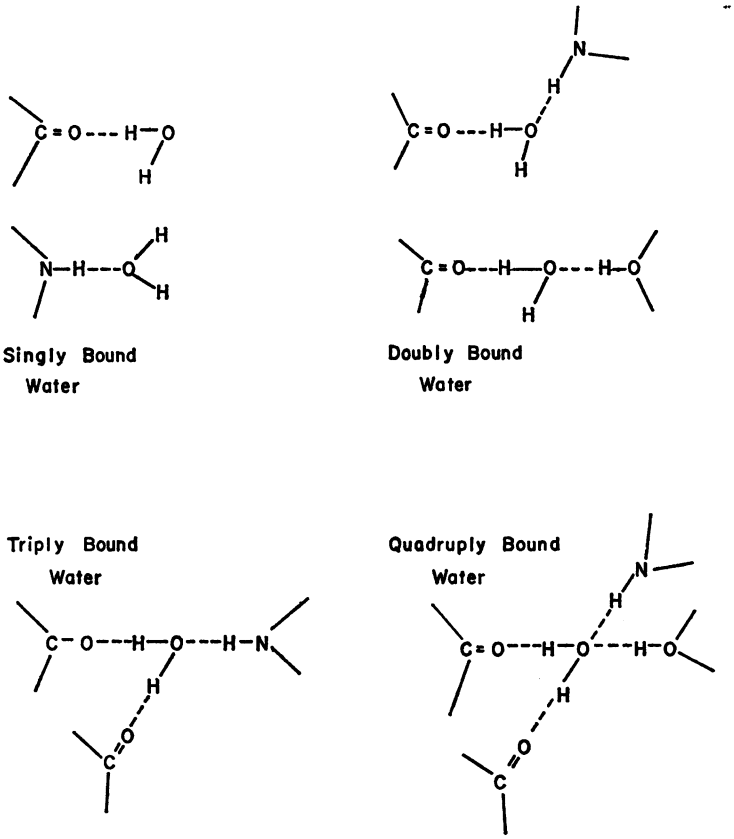

FIGURE 6 The possible types of the bound water to the peptide chains in collagen. The CO, NH, and OH groups are part of the polypeptide chains.

states (i.e., water which is singly, doubly, triply, or quadruply bonded to the residues of the peptide chains). These states are illustrated in Fig. 6. To determine the distribution of water among these five states as a function of temperature we will use the approach which Haggis et al. (16) used for pure water, appropriately modifying it to take account of the fact that unbound water can diffuse out of the system.

Let us consider the case of our continuous measurements, in which there exists unbound water at the time of measurement. For a given bond the probability of its breaking and forming another is given by

$$m_i f_1(T) e^{-(\Delta H/RT)} = n_{i-1} f_2(T) \qquad i = 1, 2, 3, 4, \tag{1}$$

where n_i is the number of i-bonded-type water molecules, that is, water molecules which form i-bonds with the peptide chain, in a given volume, m_i is the correspond-

ing number of i-type bonds, and ΔH is the bond strength per mole. Further using the fact that the sum of the n_i must be equal to the total number of water molecules per unit volume, we can write

$$\sum_{i=1}^{4} n_i = N, \tag{2}$$

where, because of diffusion, N is a function of both the time and the temperature. For a given molecule, we have

$$\begin{aligned} in_i &= m_i \\ i &= 1, 2, 3, 4. \end{aligned} \tag{3}$$

Finally we can write

$$\sum_{i=1}^{4} m_i = C(1 - P)N, \tag{4}$$

which expresses the fact that the total number of bonds is equal to the average number of bonds per molecule (C) multiplied by the fraction of bonds which are not broken (P is the fraction of bonds broken at a given temperature) multiplied by the number of water molecules present. These are modified equations used by Haggis et al. (16).

If we take the ratio $[f_1(T)/f_2(T)]e^{-(\Delta H/RT)}$ as one unknown, we will have 10 equations in 11 unknowns. In order to solve these equations we must determine N, either experimentally or theoretically. Experimentally N was determined from the weight loss as a function of temperature as measured in our TGA experiments (see Fig. 5).

For the solutions of the above equations we must also know P as a function of temperature. We approach this by treating the loss of water as an order-disorder transition of the type first developed by Bragg and Williams (17) and later by Bethe (18). Following Bethe, let us define the disorder parameter as

$$\sigma = 1 - \tfrac{1}{2}\langle n \rangle, \tag{5}$$

where $\langle n \rangle$ is the average number of polar groups of the chains near a central water molecule. In order to determine $\langle n \rangle$ we must know the structure of the crystal that we are considering. The structure of water in collagen is still in the speculative stage, but it is reasonable to assume, as does Berendsen (9), that the water will have approximately tetrahedral coordinates. With this structure each central water molecule can form bonds with up to four nearest neighbor polar groups. These polar groups can be either parts of the polypeptide chains or other water molecules to which the central water is hydrogen bonded.

Using these coordinates we may evaluate the average number of polar groups as

$$\langle n \rangle = \frac{\sum_{n=0}^{4} nP_n}{\sum_{n=0}^{4} P_n}, \tag{6}$$

where P_n is the probability of n-type bonding. If the bond strength is V we can write the total probability as

$$Z = \sum_{n=0}^{4} P_n = \sum_{n=0}^{4} \binom{4}{n} e^{-nV/KT}. \tag{7}$$

Thus, we have

$$\sigma = \tanh \frac{V}{2KT}. \tag{8}$$

In deriving the above equation we have neglected interactions beyond nearest neighbors since they should be small and there is much greater probability that second neighbors are not polar.

Assuming that $V = V_0\sigma$ (17), an assumption that has been successfully used in the past, we can iteratively solve for σ in equation 8. Using this disorder parameter we can then evaluate the fraction of bonds broken as a function of temperature from

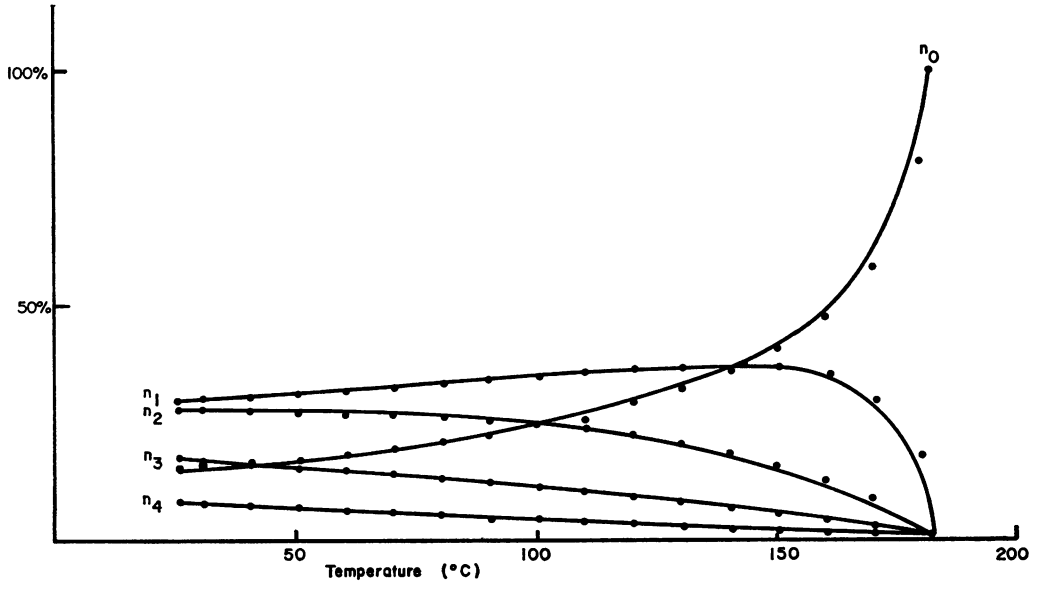

FIGURE 7 n_i, the number of i-type bonded water in a unit volume, as a function of temperature for a completely dried sample (zero water content; kept for 24 hr at 130°C).

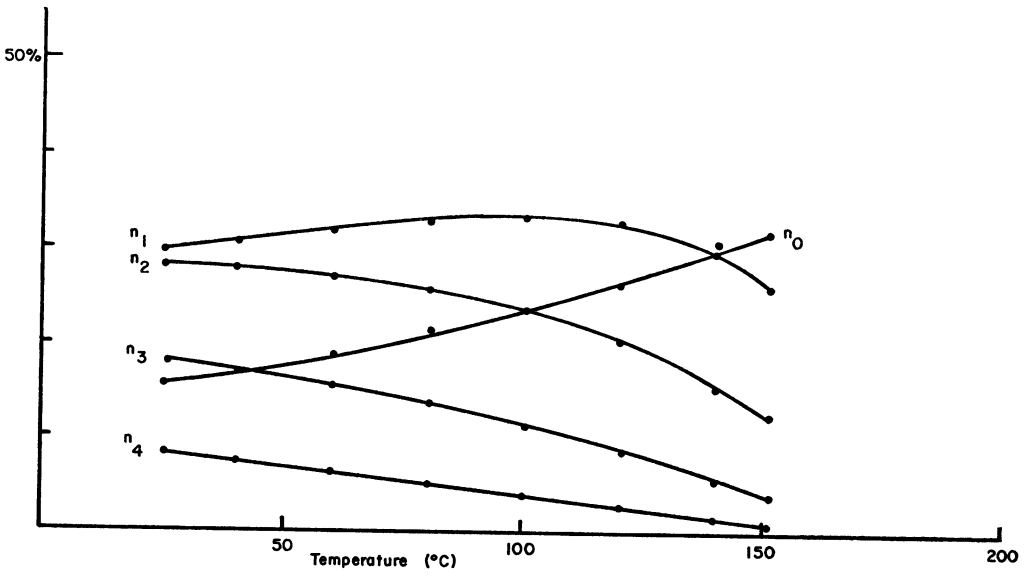

FIGURE 8 n_i, the number of i-type bonded water in a unit volume, as a function of temperature for a vacuum-dried sample (dried 2 wk) under 30 μ pressure.

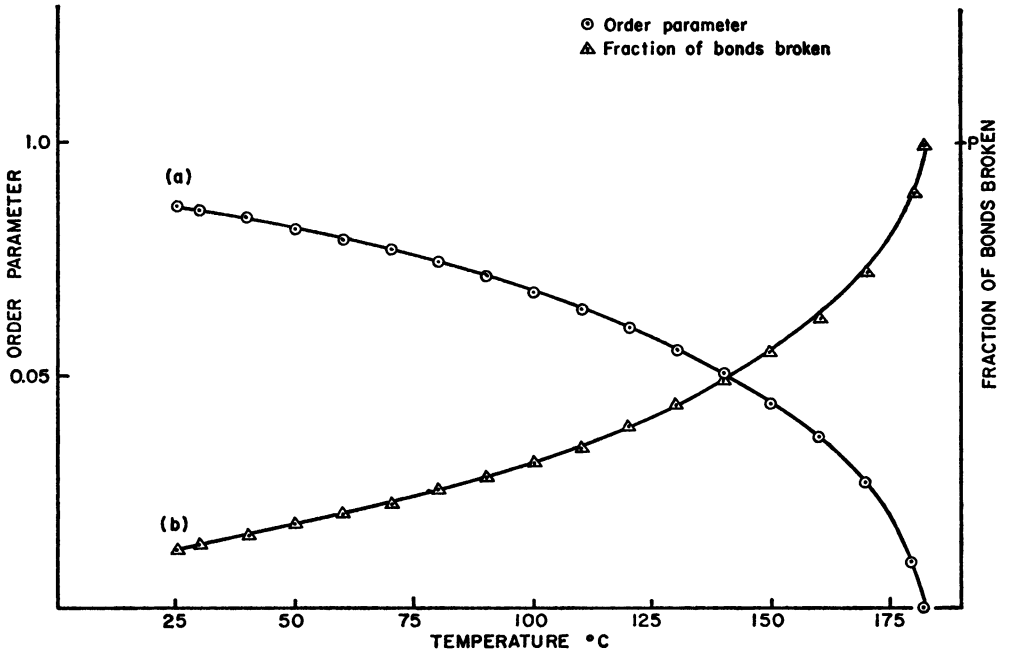

FIGURE 9 (*a*) The disorder parameter as a function of temperature. (*b*) Fraction of bonds broken as a function of temperature.

the definition used by Haggis et al. (16)

$$P = \left(1 - \frac{V}{V_0}\right) = (1 - \sigma). \tag{9}$$

P and σ have been plotted as a function of temperature in Fig. 9. As can be seen from this graph, most of the bonds are broken at temperatures above 100°C. It should also be noted that at room temperature the system is in a disordered state, a conclusion which agrees with the observation that at room temperature it takes an extremely long time to remove the free water.

For the samples with epoxy electrodes that were dried at 130°C for 24 hr, the TGA measurements show that the water loss during reheating is negligible and hence N can be taken as being constant. The solutions to the above equations (1–4) for constant N are shown in Fig. 7. For samples with a high water content, or which are heated slowly, the loss of water becomes significant; hence the value of N obtained from TGA measurements must be used in solving these equations. Using this N we obtain the values shown in Fig. 8 for n_i. If we compare these figures (Figs. 7 and 8) with Figs. 3 and 4, which show the dielectric constant, the agreement is very good.

CONCLUSIONS

Thus our results indicate that:

(*a*) Although the water and the individual residues of the peptide chains are highly polar, the bonded structure in which they exist in natural tendon is effectively nonpolar. Consequently the dielectric behavior of tendon is determined by the polar unbound water that is present. Further, since below the melting temperature of collagen no cooperative transitions occur, neither the water nor the polar groups of the peptide chains can contribute to dielectric absorption.

(*b*) The bound water in collagen exists in any of several bonded states, a significant portion of the water being multiply bonded even at relatively high temperatures.

(*c*) For a given initial concentration, the concentration of water in collagen matrix as a function of temperature is closely related to an order-disorder type of transition.

(*d*) If we take into account the possibility of the formation of water-water bonds, the result would be different. This may be done by the appropriate modification of equations 1–4. This modification becomes important only at water concentrations above 20% and has been omitted in our work.

(*e*) As a more exact model for the structure of collagen becomes available, improvements may be made in the assumed bonding structure of the water in collagen matrix and consequently a better evaluation of the disorder parameter should be possible.

(*f*) Finally, for future work, the dielectric measurements of solid tendon must be

extended to the low frequency limit, i.e., to the static case. Further dielectric measurements of collagen in solution are necessary to fully understand the role of water and of the collagen macromolecule.

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