A REACTION OF WATER WITH RAT-TAIL TENDONS (HYDRATION-ELONGATION)

HARRY R. ELDEN AND ROBERT J. BOUCEK
Howard Hughes Medical Institute, Miami, Fla. (U.S.A.)
(Received May 5th, 1959)

SUMMARY

1. The rate of lengthening of a dry tendon was analyzed when water, water–salt, and water–alcohol solutions were added.

2. An empirical-mathematical treatment of the experimental data resulted in a certain constant (K_{HE}) which could be related to the temperature ($1/T$), dielectric constant (D) and ionic strength (μ) of the water solutions.

3. The experimental findings were compared with recent knowledge of the interaction of water with proteins. Also, the influence of the solvent was examined in light of the possible existence of electrostatic stabilization forces in the collagen-mucopolysaccharide interaction of tendons.

INTRODUCTION

It is recognized widely that water has an important role in biological systems. In a recent review of this subject KLOTZ[1] supported the idea that "lattice" water was bound intimately to proteins. This was suggested to explain the masking of functional groups of protein *molecules in solution.*

Collagen is a fibrous protein in close union with water, carbohydrates and amino sugars (mucopolysaccharides). According to FESSLER[2] mechanical stress, shock, and trauma might be transmitted through sets of "sponges" composed of water-mucopolysaccharide molecules and the fibrous constituents. These sponges also could account for the viscoelastic properties of connective tissue and other hydrated systems. The central theme of the studies in our laboratory is to determine how the elasticity and swelling of rat tail tendons might be influenced by water. From this it may be possible to study the cross-linking reactions which collagen appears to undergo as a result of the process of aging[3–5]. It is the purpose of this report to present a mechanico-chemical analysis of the *hydration-elongation* of tendons and to study the effect of mixed solvents and salt solutions upon this process.

EXPERIMENTAL

Preparation of tendon

Tendons were removed from rat tails after the skin was cut the full length with scissors and peeled away by hand. A break between the vertebrae was made with

a pair of pliers and the attached tendons were pulled from the rest of the tail. Proceeding from the tip, tendons were removed successively. Lengths shorter than 12 cm were not used. The collection of tendons was washed 3 times with 50-ml portions of 0.1 *M* sodium chloride, then washed with distilled water for 30 min. They were removed, disentangled, and dried. Tendons dried to a constant weight/length (W) within 30 min following exposure to the atmosphere of the laboratory (22° to 23° temperature and 75 % relative humidity). More extensive drying was attempted but subsequent handling caused some rehydration thereby re-establishing the original dry weight/length ratio (W).

Apparatus

The tendon was inserted into a 20-ml syringe cylinder through the bore of the glass tip; one end was immobilized by forcing an adapter (2 or 3-way) over the tip while the other end, 10 cm distant, was attached to a mechanical lever system by a small "alligator" clip. A meter stick was suspended and balanced on metal knife edges and provided a writing lever arm (70 cm long) and a driving arm (3.5 cm long) which caused a 20× magnification. Records of length *vs.* time were obtained by an electrically driven kymograph. The system was unbalanced slightly so that there was a positive load of 20 g on the tendon. A vacuum line was attached to one of the ports of the adapter on the syringe and air was sucked through the cylinder. This facilitated removal of liquids and was a satisfactory means of drying the tendon between repeated runs.

Hydration

The tendon was hydrated by rapidly (1–2 sec) admitting liquid of volume sufficient to cover it. The liquid that was added and the syringe-tendon assembly were held at constant temperature in a thermostated water bath. The temperature dependence of the hydration-elongation was studied in water bath temperatures ranging from 10 to 60°.

Dehydration

The temperature of dehydration was varied by using a hair dryer to force air into the cylinder. Regulation was achieved by alternating the heater switch from ON to OFF while the exact temperature was indicated by a thermometer placed alongside the tendon in the cylinder. In this manner the drying and concomitant shortening of the tendon was at air temperatures ranging from 20 to 100°. The influence of the rate of delivery of air at a fixed temperature was measured. The current to the fan of the dryer was varied by using a Variac and the shortening was measured at Variac settings from 110 to 0 at a temperature of 23°.

Influence of tendon thickness

The influence of tendon thickness was studied by measuring the hydration-elongation of specimens having W values from 0.1 to about 0.6 mg/cm.

Mixed liquids

The hydration-elongation was studied in water and its binary mixtures with methanol, ethanol, isopropanol, and propanol. The composition of the mixes ranged

from 0 to 100% alcohol. All of these studies were done at a temperature of 23°. Dielectric constant data were obtained from the literature[6] for the solutions described.

Salts

The influence of ionic strength of sodium chloride was studied by making up solutions ranging from 0 to 1.0 *M*. Four tendons with W values ranging between 0.2 and 1.0 mg/cm were used for this study.

RESULTS

Fig. 1 shows a typical length–time curve. Upon the addition of water the length $(L_0 + L)$ of the tendon increased from the initial "dry" value (L_0) to a steady-state value $(L_0 + L_\infty)$ in about 1 min. At this point the water was removed and air was sucked through the cylinder. The tendon was dried at a temperature of 23° until it ceased to shorten. The "dry" length was called L_0 and the maximum increase in length (L_∞) was a distance of about 2–4 mm. The length *vs.* time curves produced by subsequent addition of water followed by drying could be reproduced at least 10 times.

There was a progressive increase in shortening when the dehydration was conducted at higher air temperatures. The extent of shortening was constant for Variac settings from 110 to 80. Below this the rate of delivery of air became rate limiting and inadequate drying resulted.

Fig. 2 shows the shape of length *vs.* time curves obtained when the drying temperature was increased from 21 to 100°. $L_0 + L_\infty$ did not change but L_0 was reduced. A particular length *vs.* time curve could be reproduced when the temperature of the hydrating liquid and the air-dehydration were reproduced. The two temperatures need not be the same. Once a tendon was heated a curve previously obtained at a lower temperature was shifted to high length when the elongation was done at the lower temperature. In this way the dehydration was not reproducible. But, if the temperature of the two steps were repeated the hydration-elongation and dehydration-shortening could be reproduced.

The rate of elongation $(\Delta L/\Delta t)$ was calculated and appears as a function of t

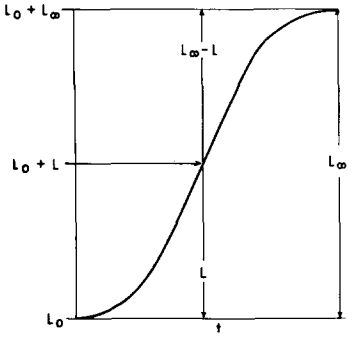

Fig. 1. A typical length–time curve illustrating the hydration-elongation of rat tail tendons. The symbols identify those aspects of the process which are used in the analysis presented in the text.

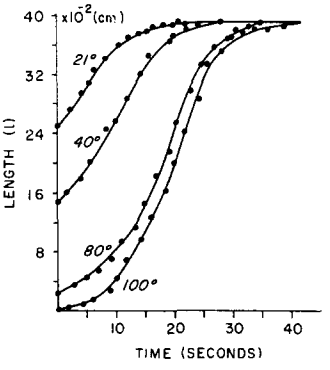

Fig. 2. The influence of dehydration-temperature upon the subsequent hydration-elongation conducted at 25°.

in Fig. 3. The maximum in the velocity of elongation always was sharp. When the tracings were made at very fast speed the break in the curve continued to be sharp.

The velocity ($\Delta L/\Delta t$) was plotted against ($L_\infty - L$) and curves were obtained that were similar to those shown in Fig. 3. However, Fig. 4 shows that plots of $\Delta L/\Delta t$ *versus* the product $(L_\infty - L)t$ were linear with a positive slope (K_{HE}). This empirical observation formed the basis for eqn. (1).

$$dL/dt = K_{HE}(L_\infty - L)t \qquad (1)$$

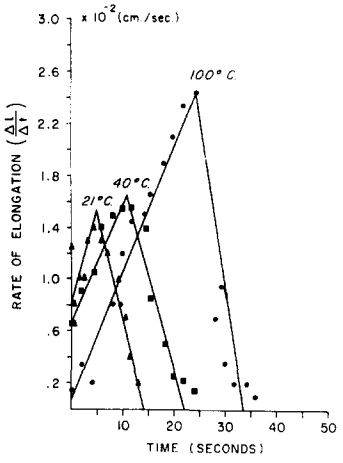

Fig. 3. An empirical analysis of the velocity of elongation as a function of time and dehydration temperature.

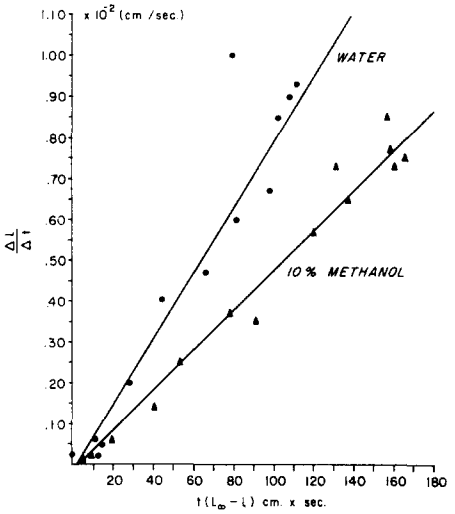

Fig. 4. The linear relationship between velocity of elongation and the quantity $t(L_\infty - L)$ as influenced by water and 10% methanol.

Here, dL/dt is the rate of change of the length of tendon during the hydration, K_{HE} is a specific rate constant for the process, L_∞ is the maximum increase in length attained, and L is the change in length at any particular time, t. Rearrangement of the terms in equation (1), followed by integration between the limits of 0 to L in the time interval of zero to t, gives eqn. (2).

$$\text{Log}(L_\infty - L) = \log(L_\infty) - K_{HE}t^2/(2 \times 2.303) \qquad (2)$$

Data obtained previously for the elongation of tendons by water[7] were in complete agreement with eqn. (2).

The influence of the tendon thickness was studied by comparing K_{HE} and W evaluated from data obtained with water. Fig. 5 shows that log K_{HE} was related linearly to log W with a slope of -1.

The velocity at which the tendon elongated in water, denoted by the specific constant K_{HE}, was dependent upon the temperature. Eqn. (3) shows the equation which was found to relate the constant K_{HE} and the reciprocal of the absolute temperature ($1/T$).

$$\text{Log } K_{HE} = A - B\ (1/T) \qquad (3)$$

The hydration-elongation of tendons induced by binary liquid systems showed some interesting effects. Plots of log $(L_\infty - L)$ *versus* t^2 for alcohol–water solutions

showed an initial downward curvature which eventually became linear with t^2. When K_{HE} was evaluated from this linear region it could be related to the dielectric constant of the medium by eqn. (4). The methanol–water system deviated the least from eqn. (2) and was in agreement with eqn. (4) from 0 to 40 % methanol.

$$\text{Log } K_{HE} = \log (K_{HE})_0 + C\,(D - 1)/(2D + 1) \qquad (4)$$

In the non-linear region of eqn. (2) there appeared to be a systematic way in which the deviations occurred. Values of t^2 which reduced the y-ordinate to one-half ($\frac{1}{2}$) the value at zero time were estimated from graphs of log $(L_\infty - L)$ *versus* t^2. The values of $t^2_{\frac{1}{2}}$ for solutions with a dielectric constant of 50 formed the series Me(2000), Et(380), Isopro(200), and Pro(100).

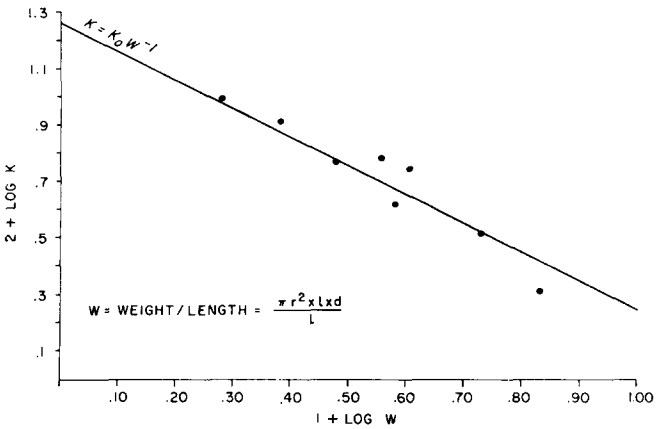

Fig. 5. The dependence of the specific constant (K_{HE}) upon the size (W) of rat-tail tendons.

Eqn. (5) shows the relationship that was used to relate the rate constant (K_{HE}) to the ionic strength (μ).

$$\text{Log } K_{HE} = \log (K_{HE})_{\mu = 0} - E(\sqrt{\mu}) \qquad (5)$$

DISCUSSION

Water

GUSTAVSON, in his excellent treatise on the chemistry of collagen[8], summarized the current thinking on its hydration. The sites wherein water becomes attached to collagen might be (a) the polar side chains and (b) the oxygen and nitrogen atoms of the peptide bond. The ionic groups (NH_3^+) and (COO^-) might also bind water but since they are so few in number the hydration cannot be attributed largely to them. Mucopolysaccharides also bind water and FESSLER[1] pointed out important viscoelastic properties of the hydrated collagen-mucopolysaccharide system.

According to KANAGY[9], WOLLENBERG[10], and ROUGVIE[11] water is adsorbed by collagen in a succession of steps. The most reactive sites attach water first while those of less binding ability become involved later. BULL[12] showed that about 21 % of the water of hydration in collagen was tightly bound whereas the 79 % of water subsequently added forced the chains apart so that their separation increased from 10 Å to 15–16 Å. The discontinuous adsorption of water probably holds for mucopolysaccharides as well.

In the dehydration step of the system which we described here the tightly held water probably was not removed. The subsequent addition of water, which permitted the hydration-elongation to occur, conceivably allowed the collagen-mucopolysaccharide chains of tendons to separate. At high drying temperatures more water was removed and tendons shortened more than at low drying temperatures.

Rat tail tendons undergo considerable swelling when placed in water. A model-mechanism that was consistent with the volume–time data was proposed by ELDEN[13] to account for the swelling of hide powder collagen and rat tail tendons. Eqn. (6) shows the proposed relationship between the initial volume (V_0), the final volume (V_∞), a specific swelling constant (K_s), a constant characteristic of the ability to resist any increase in volume (a), and the accumulated time of swelling (t).

$$V^2 = V_\infty^2 - (V_\infty^2 - V_0^2)\, e^{-2aK_s t} \tag{6}$$

When a 20-g tension was added to the tendon, comparable to that involved in the hydration-elongation, the tendon did not swell as much as without the load[14]. The longitudinal force probably stretched the tendon so that lateral expansion was not accomplished easily.

Let us inquire as to whether or not the rate of elongation was dependent upon the amount of water which the tendon has imbibed. To do this it is assumed that the volume (V) can be replaced by the term Area $\times$ L. If this is inserted for V_∞ and V with V_0 equal to zero, eqn. (6) becomes eqn. (7).

$$\text{Log}\,(L_\infty - L) = \log \frac{(L_\infty)}{(L_\infty + L)} - 2aK_s t \tag{7}$$

This is not comparable with eqn. (2) and does not decrease rapidly enough to coincide with the experimental points. Thus, it appears that there is a rapid reaction between water molecules and those centers which hold the tendon in its shortened state. This reaction goes to completion rapidly and the subsequent imbibition of water by swelling has no effect on the elongation.

Mixed solvents and salt solutions

The influence of solvent properties on the rate of simple ion-ion, ion-dipole, and dipole-dipole reactions in dilute solutions can be expressed by eqn. (4). When the dielectric constant becomes small, positive deviations of experimental data occur and this has been attributed to participation of the solvent in the reaction[15].

Specific solvent effects do appear to be involved when alcohol–water solutions were used for hydration-elongation. The deviation of the plots according to eqn. (2) became progressively greater in comparison to the series Me < Et < Isopro < Pro alcohols. Likewise, the electronegativity around the hydroxyl group[16], heats of hydration[17], and VAN DER WAALS' constants also increase in the same order. We do not know what part of the collagen-mucopolysaccharide system has an affinity for these alcohols.

The equations which show the dependence of K_{HE} on (D), and (μ) are identical in form to those kinetic expressions which account for the rate of interaction of charged molecules in solution[15]. These findings suggest that analogous electrostatic processes might participate in the solvent-induced lengthening of tendons.

These findings agree with those of BENSUSAN who measured the rate of formation

of collagen fibers from soluble molecules[18]. The rate depended upon the concentration of alcohols used to change the dielectric constant. In the concentration region of inhibition the alcohols effectiveness increased in the order Me < Et < Isopro < *tert.*-Butyl < Pro. Increased ionic strength of simple electrolytes also decreased the rate of fiber formation.

At the isoelectric point of native collagen (pH 7) there are equal numbers of cationic and anionic charges. However, there could be an un-equal distribution of charges in a local region which could attract, or repel, similar excess local charges. The close proximity of mucopolysaccharides to collagen favors this situation because sulfonic acid groups of the former are dissociated at neutral pH. This could regulate the rate and extent of hydration-elongation by restricting, or enhancing, the separation of these chains.

It is hoped that an elucidation of the nature of these charged sites will contribute to our knowledge of collagen-mucopolysaccharide stabilization. Then, the participation of these sites in the aging of tendons might be examined.

ACKNOWLEDGEMENTS

We wish to express our appreciation to Drs. N. L. NOBLE, W. B. DANDLIKER, and J. F. WOESSNER, Jr. for their many stimulating thoughts and suggestions. The technical skills of Mrs. P. R. PORTER were of great assistance to us.

REFERENCES

1 I. M. KLOTZ, *Science*, 128 (1958) 815.
2 J. H. FESSLER, *Nature*, 179 (1957) 426.
3 I. BANGA, J. BALO AND D. SZABO, *Experimentelle Alternsforschung*, Birkhauser Verlag, Basel, 1956, p. 28.
4 W. G. BANFIELD, *J. Gerontol.*, 11 (1956) 372.
5 R. R. KOHN AND E. ROLLERSON, *J. Gerontol.*, 13 (1958) 241.
6 H. S. HARNED AND B. B. OWEN, *The Physical Chemistry of Electrolytic Solutions*, Reinhold Publishing Corporation, New York, N.Y., 2nd ed., 1950, p. 118.
7 H. R. ELDEN, *Nature*, Submitted for publication.
8 K. H. GUSTAVSON, *The Chemistry and Reactivity of Collagen*, Academic Press Inc., New York, N.Y., 1956, p. 147.
9 J. R. KANAGY, *J. Am. Leather Chemists' Assoc.*, 45 (1950) 12.
10 H. G. WOLLENBERG, *J. Soc. Leather Trades' Chemists*, 36 (1952) 172.
11 M. A. ROUGVIE AND R. S. BEAR, *J. Am. Leather Chemists' Assoc.*, 48 (1953) 735.
12 H. B. BULL, *J. Am. Chem. Soc.*, 66 (1944) 1499.
13 H. ELDEN, *Science*, 128 (1958) 1624; 183 (1959) 1681.
14 H. R. ELDEN, P. R. PORTER AND R. J. BOUCEK, Presented at *Meeting-in-Miniature, Florida Section, American Chemical Society, St. Petersburg, Florida, May, 1959.*
15 S. GLASSTON, K. J. LAIDLER AND H. EYRING, *The Theory of Rate Processes*, McGraw-Hill Book Company, 1941, p. 400.
16 M. J. S. DEWAR, *The Electronic Theory of Organic Chemistry*, Oxford University Press, Amen House, London. 1st ed., 1949 (Reprinted 1950), p. 51.
17 J. A. V. BUTLER, *Trans. Faraday Soc.*, 33 (1937) 229.
18 H. B. BENSUSAN, *Abstract of Papers, 134th Meeting, American Chemical Society, New York, N.Y., September 1958.*