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NUCLEAR MAGNETIC RESONANCE STUDY OF COLLAGEN HYDRATION

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TECHNICAL REPORT 398

JULY 2, 1962

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Nuclear Magnetic Resonance Study of Collagen Hydration*

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(Received November 10, 1961)

The proton resonance signal of water in partially dried tendon shows three peaks for samples equilibrated in an atmosphere of 30%–80% relative humidity (R.H.). The outer-peak splitting (between 0.5 and 1 gauss) has the character of a proton-pair splitting with effective interactions in, or close to, the fiber direction, as concluded from the dependence of the absorption on the angle between the fiber direction and the magnetic field. Chainlike water structures, exhibiting certain proton-reorientation processes, are postulated. At 10% R.H. a narrow peak is observed, which is not angular dependent; this indicates rapid reorientation of water molecules. Above 80%–90% R.H., a single peak is observed, the width of which has a strong angular dependence. A structural relationship between collagen and water exists, by virtue of which the existence of water chains and, possibly, of three-dimensional water structures, could be stabilized.

I. INTRODUCTION

THE existence of icelike structure in the water of hydration has been proposed[1-5] for proteins,[3] deoxyribonucleic acid (DNA),[4-5] and other biological macromolecules.[5] Structural organization of water near macromolecules is of interest with respect to several functions, such as ionic selectivity in cells[6] and energy transfer in proteins.[7] Configurational changes of polypeptides induced by water have been reported,[8] and a theory of interaction between protein configuration and water structure might connect the aforementioned ideas[6] with the denaturation theory of diffuse excitation.[9]

Nuclear magnetic resonance (NMR) measurements have been interpreted as indicating that part of the water in DNA solutions[10] and solutions of Tobacco Mosaic Virus (TMV)[11] is in "icelike" form and gives no contribution to the area under the narrow water peak. More recently, however, it has been suggested[12] that the results of these measurements were influenced by experimental errors attributable to an inappropriate rate of passage through the resonance conditions.[13] Subsequently, no significant decrease in area under the water peak has been observed for solutions of DNA[12] and TMV.[14] A line broadening that was observed in these cases, as well as for agar–agar gels,[15] has been attributed to diamagnetic inhomogeneities in the neighborhood of π-electron systems,[12] to paramagnetic effects and other causes,[14] and to structural organization of water.[15]

Our study is devoted to the hydration of collagen, which has the following advantages not shared by most other proteins: (a) it is available in reasonably pure state in native tissue; (b) it can be dried to a high degree presumably without losing its macromolecular structure, and thus permits investigation over a broad range of water content; (c) it is available in oriented fibrous form; thus additional information can be gained from the study of the NMR absorption as a function of the angle between fiber and field.

II. EXPERIMENTAL

Bovine Achilles tendon was used throughout the measurements. The collagen content of this tissue is approximately 85% of the dry weight,[16] the rest consisting mainly of elastin (4.9%[16]) and polysaccharides.

Sections with reasonably parallel orientation were cut into cylindrical pieces with the fiber direction perpendicular to the cylinder axis. Samples were equilibrated in atmospheres of constant humidity by storage for three weeks at 20°C over saturated salt solutions[17] in closed vials, and subsequently were put into test tubes, sealed, and stored at 5°C until they

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10 B. Jacobson, W. A. Anderson, and J. T. Arnold, Nature 173, 772 (1954).
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13 R. B. Williams, Ann. N. Y. Acad. Sci. 70, 890 (1958).
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16 R. E. Neuman and M. A. Logan, J. Biol. Chem. 186, 549 (1950).
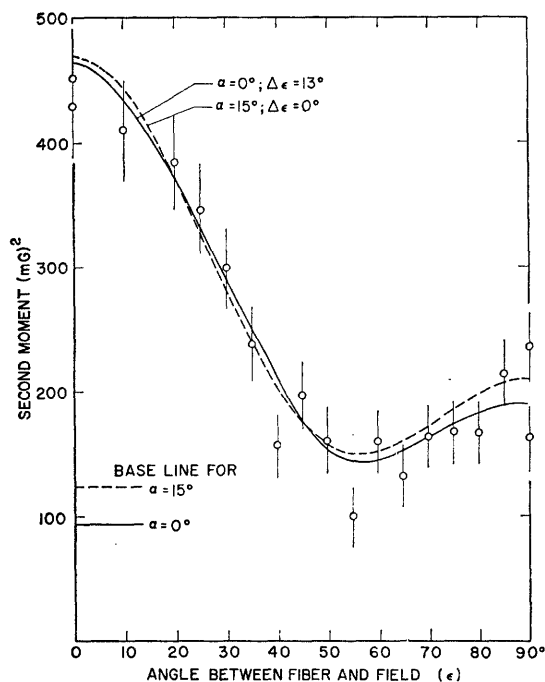17 Salt solutions were chosen from a list given by F. E. M. O'Brien, J. Sci. Instr. 25, 73 (1948).

FIG. 1. Second moment of the proton resonance signal of sorbed water in native-state tendon as a function of the angle ϵ between the magnetic field and the fiber direction. Only contributions over 200 mgauss were taken. Solid curve gives the theoretical angular dependence of the second moment for proton interaction in the fiber direction with a Gaussian distribution of fiber directions having a standard deviation of 13°; dashed curve is the theoretical curve for proton interactions at an angle of 15° with the fiber axis. A constant value has been added to both curves as indicated by the base lines.

were used for measurements. Equilibration was monitored by weight measurements and was virtually completed after two weeks. The weight of the samples varied between 0.5 and 1 g.

Initial measurements were carried out with larger samples in the native state on a Schlumberger NMR analyzer model 104 at 7.3 Mc/sec; all measurements with equilibrated samples were carried out on a Varian model V4250 low-resolution NMR spectrometer, operated at a frequency of 60 Mc/sec, and in combination with a Varian 12-in. magnet system. The samples could be rotated in the field and their position read on on an angular scale. Field modulation of 40 cps was used, and the signal from the low-frequency phase detector was electronically integrated by an integrator with long time constant. The magnetic field was swept through resonance in approximately 2 min. Radio-frequency intensities were 10 db or more below the intensity for which the absorption signal has its maximum value. The rate of the slow field sweep was calibrated by modulating the 60-Mc/sec oscillator with a low-frequency sine wave of known frequency, thereby producing known sideband frequencies at which absorption occurs. Measurements were taken at room temperature (21°–24° C).

III. RESULTS

Initial measurements with the Schlumberger NMR analyzer on untreated tendon show a single water line with a width between inflection points of less than 25 mgauss, varying appreciably with the angle ϵ between fiber direction and magnetic field. The peak-to-peak amplitude of the derivative signal is strongly angular dependent, and shows a sharp maximum for a fiber-field angle of $(55.0\pm0.5)°$ and a minimum for 0_1°. An amplitude ratio of five between the angles 55° and 0° was commonly observed. This seemed to indicate that the protons interact effectively in the fiber direction, since proton interaction cancels for an angle of 54°44′ between the field vector and the vector connecting two interacting nuclei.

To test this further, the second moment S of these same curves[18] was plotted for one sample as a function of the fiber-field angle ϵ (circles in Fig. 1). The second moment S is defined as

$$S=\left[\int_0^\infty (H-H_0)^2 g(H)\,dH\right]\Big/\left[\int_0^\infty g(H)\,dH\right], \qquad (1)$$

where $g(H)$ represents the absorption as a function of the magnetic field strength H, and H_0 is the field at the center of the absorption line. One can calculate S for a cylindrically symmetrical system in which effective interaction of the nuclei takes place only in directions at an angle α with the cylinder (fiber) axis. An example of such a system is a system of isolated motionless proton pairs with the internuclear vector at an angle α with the field, and with a rotation-symmetric distribution of proton pairs around a given axis. Other examples are a rotation-symmetric distribution of linear arrays of motionless protons with negligible interaction between different linear arrays, or a system of isolated groups of protons, in which the protons in each group reorient in such a way as to have a resulting interaction at an angle α with the symmetry axis. Since independent interactions simply give an additive contribution to the second moment, the angular dependence of S is found by calculation of

$$[(3\cos^2\theta-1)^2]_{Av},$$

where θ is the angle between the direction of interaction and the magnetic field, which is a function of the three angles α, between the direction of interaction and the symmetry axis, ϵ, between the symmetry axis and the magnetic field, and ϕ, the azimuthal angle of the direction of interaction. The average has to be taken over all azimuthal angles ϕ.

It is then found that

$$A\,S(\alpha, \epsilon) = (315\cos4\alpha+180\cos2\alpha+81)\cos4\epsilon$$
$$+(180\cos4\alpha+432\cos2\alpha+156)\cos2\epsilon$$
$$+(81\cos4\alpha+156\cos2\alpha+467). \qquad (2)$$

[18] In order to avoid large errors and appreciable contributions of the protons in the collagen molecules, the second moment was taken over 200 mgauss. This may limit the validity of the results.

Here, A is a constant depending on specific geometry and motion of the nuclei. For isolated proton pairs with an interproton distance r, the value of A is given by

$$A = 2048r^6/9\mu^2,$$

where μ is the proton magnetic moment. A representation of $S(\alpha, \epsilon)$ is given in Fig. 2, in which the scale values on the ordinate apply to a system of motionless proton pairs.

It is emphasized that the study of the angular dependence of the second moment of fibrous materials is very useful for obtaining information about the directions in which interactions take place.

A distribution of angles ϵ, because of imperfect alignment of fibers, will influence $S(\alpha, \epsilon)$. For $\alpha = 0°$, with

$$S(0, \epsilon) \propto (3 \cos^2\epsilon - 1)^2, \tag{3}$$

one obtains for a symmetric distribution of angles ϵ, with mean ϵ_0:

$$\langle (3 \cos^2\epsilon - 1)^2 \rangle = (3 \cos^2\epsilon_0 - 1)^2$$

$$+ (18 \sin^2 2\epsilon_0 - 3 \cos 2\epsilon_0 - 9) \langle (\epsilon - \epsilon_0)^2 \rangle$$

$$+ (24 \cos^2 2\epsilon_0 + \cos 2\epsilon_0 - 12) \langle (\epsilon - \epsilon_0)^4 \rangle. \tag{4}$$

In Eq. (4), the angular brackets denote the average over the ensemble of ϵ's; ϵ and ϵ_0 are expressed in radians, and sixth and higher moments of the distribution are neglected. This function, calculated for a Gaussian distribution [for which

$$\langle (\epsilon - \epsilon_0)^4 \rangle = 3 \langle (\epsilon - \epsilon_0)^2 \rangle^2]$$

with $\langle (\epsilon - \epsilon_0)^2 \rangle = 0.05$, which is a 13° standard deviation, yields the solid curve in Fig. 1; the dashed curve represents $S(15°, \epsilon)$. For both curves a constant
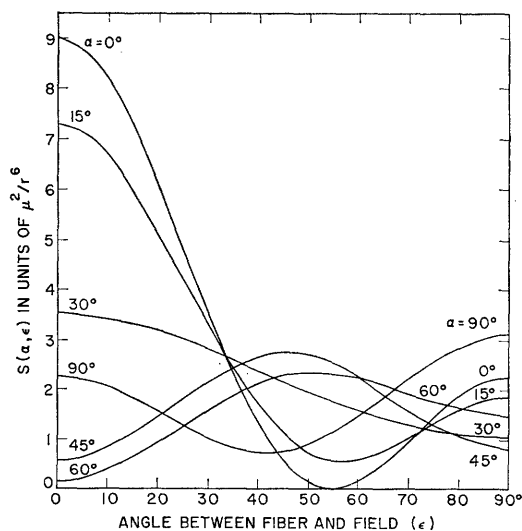

FIG. 2. Second moment $S(\alpha, \epsilon)$ of a cylindrically symmetrical system of protons interacting at an angle α to the symmetry axis, as a function of the angle ϵ between magnetic field and symmetry axis. Ordinate values are valid for isolated proton pairs.
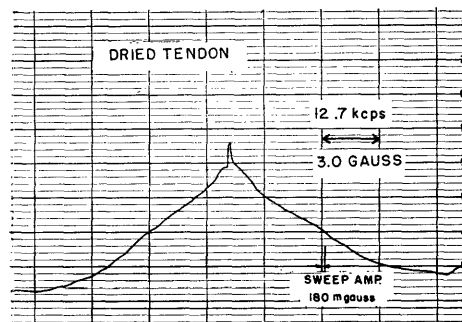

FIG. 3. Proton-absorption curve at 60 Mc/sec for tendon dried over P_2O_5. The curve was obtained by electronic integration of the derivative signal produced by the field-modulation technique. (Paper speed, 2 divisions per minute.)

value was added to obtain the best fit with the experimental data; this is justified if interactions that are not angular dependent contribute to the width of the absorption curves.

The experimental data thus can be fitted with curves for α and ϵ ranging from $\alpha = 0°$ with $\langle (\epsilon - \epsilon_0)^2 \rangle^{\frac{1}{2}} = 13°$ to $\alpha = 15°$ with $\langle (\epsilon - \epsilon_0)^2 \rangle = 0°$. Since no independent data on the spread of fiber directions are available, we can conclude that the effective proton interaction occurs in the fiber direction, or at an angle of less than approximately 15° from the fiber axis.

Measurements on partially dried samples show a water-absorption curve that can easily be separated from the collagen absorption. Figure 3 shows the absorption of a sample that had been dried over P_2O_5; its width is 8.2 gauss at half-amplitude. The absorption peak of hydration water is less than 2 gauss wide and was studied separately.

Figure 4 gives the water-absorption curves for a sample equilibrated at 32% relative humidity (R.H.), for various angles ϵ between fiber and field. The separation of the outer peaks follows $|3 \cos^2\epsilon - 1|$ within the inaccuracy resulting from the spread of fiber directions.

The curves of Fig. 4 are typical for the R.H. range of approximately 30%–80%. Figure 5 shows the line shapes for various values of R.H., for all $\epsilon = 0°$. At 10% R.H. one narrow peak is observed (note different scale units on abscissa), which shows very little dependence on the angle ϵ. At R.H. above approximately 90% no peak separation is observed; the width, however, is strongly dependent on ϵ. From this and from observations of the temperature dependence of the absorption it is seen that the ratio between the areas under the central and outer peaks depends on both relative humidity and temperature: Higher R.H. and higher temperature relatively increase the central peak. The separation of the outer peaks depends somewhat on the relative humidity; it ranges from 0.95 gauss at 32% R.H. to 0.5–0.6 gauss near 90% R.H., for $\epsilon = 0°$.

The saturation of the three peaks was tested for a 66% R.H. sample. The imaginary part $\chi''(H_1)$ of the magnetic susceptibility, relative to its unsaturated
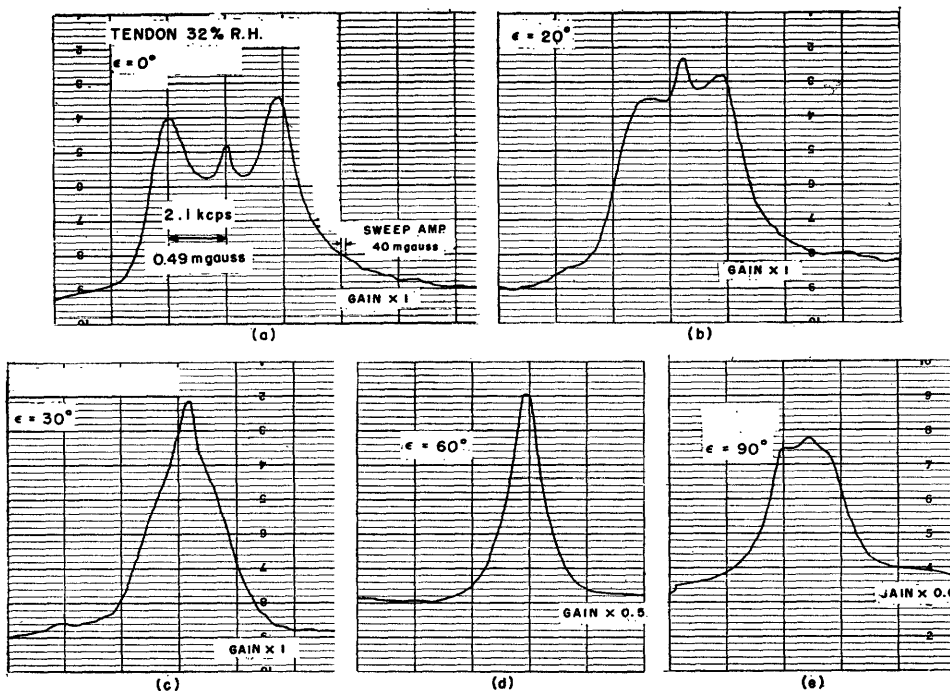
FIG. 4. Water-absorption curves for tendon equilibrated in a 32%-R.H. atmosphere for various values of the angle ϵ between magnetic field and fiber direction. (Same paper speed as in Fig. 3.)

value $\chi''(0)$, is plotted in Fig. 6 against the rf field intensity H_1, for: (a) the middle peak at $\epsilon=0°$; (b) the outer peaks at $\epsilon=0°$; (c) the single peak at $\epsilon=55°$. The solid line represents theoretical saturation be-

havior, given by

$$(1+\gamma^2 T^2 H_1^2)^{-1}, \qquad (5)$$

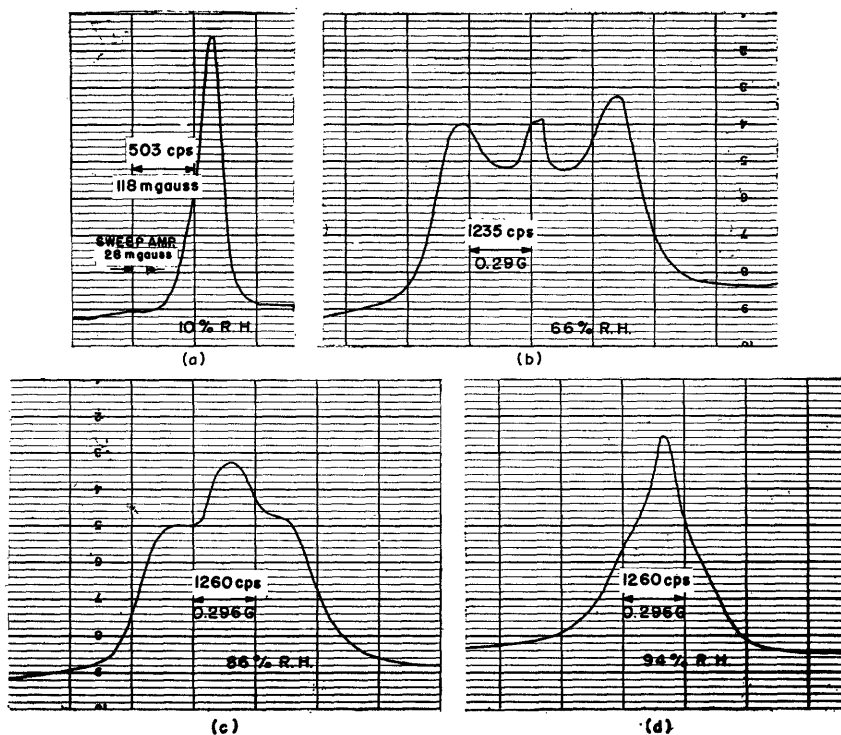where γ is the proton gyromagnetic ratio and T^2 is a

FIG. 5. Water-absorption curves for tendon with the fibers aligned with the magnetic field, for various values of relative humidity at which the samples were equilibrated. Note different scale values for 10% R.H. (Same paper speed as in Fig. 3.)

constant selected for the best fit to the experimental values. Here, T^2 is the product of the spin–lattice and spin–spin relaxation times $T_1 T_2$ for systems whose relaxation behavior can be described by two single relaxation times according to Bloch's equations,[19] or T^2 can be expressed in terms of Bloembergen's theory[20] for reorientating proton pairs, from which one can easily derive

$$T^2 = (w_1 + w_2)[w_1(w_1 + 2w_2)(w_0 + \tfrac{3}{2}w_1 + \tfrac{1}{2}w_2)]^{-1}. \quad (6)$$

The w_i's are defined by Bloembergen[20] for stochastic rotational diffusion of isolated proton pairs; the resulting value for T^2 is generally dependent on the angle ϵ.

The similarity in saturation behavior of the three peaks, as shown in Fig. 6, can be explained if the process that is responsible for the splitting of the resonance line does not contribute appreciably to T^{-2}, and the two proton species (represented by the middle peak and the outer peaks) have similar interactions with their surroundings. That two species are represented is indicated by the fact that the ratio of the area under the central peak to that under the outer peaks varies with temperature and R.H.
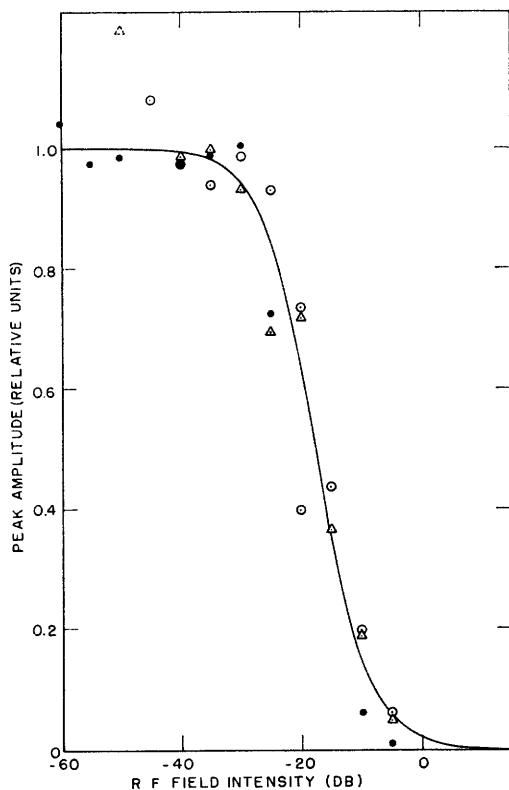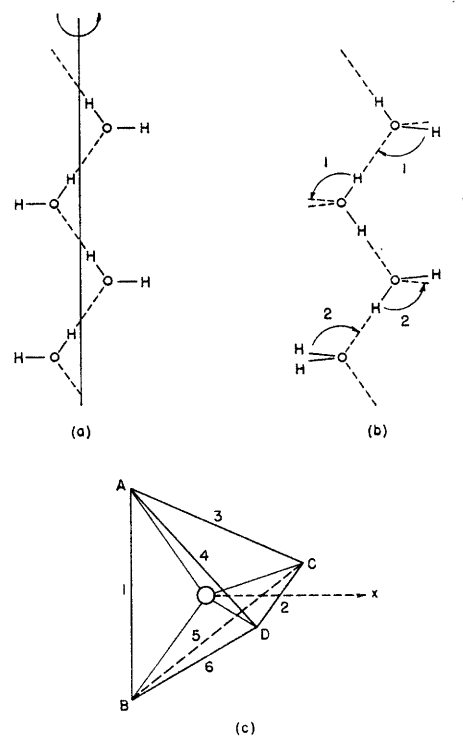

FIG. 7. Chains of water molecules: (a) structure of a chain; (b) four possible orientations of the water molecules in a chain: H(igh), V(ertical), S(ide), and L(ow), and coupled proton jumps of types 1 and 2 that produce reorientations; (c) possible proton positions A–D for a water molecule with oxygen nucleus at O. Lines 1–6 indicate directions of possible proton interactions.

In order to test whether anisotropy of the sample could cause any effects, a piece of tendon was soaked for 30 min in dioxane, and the excess liquid was removed by blotting paper. A narrow line was observed, showing no angular dependence.

IV. DISCUSSION

The magnitude and angular dependence of the observed line splitting leaves little doubt that the splitting is caused by dipole–dipole interaction. Since the central peak most likely represents a different proton species, the curves essentially have the characteristic shape of proton-pair interaction, and the assumption seems justified that the interaction between the two protons of one water molecule is responsible for the splitting. (One could visualize a rapid isotropic rotation of each water molecule, which cancels intramolecular interaction and only leaves interaction between water molecules; in such a process each water molecule would have a total spin of -1, 0, or $+1$, with probabilities of $1/4$, $1/2$, and $1/4$, respectively, i.e., it can be considered as a nuclear spin system with $I = 1$. Linear interaction of two molecules would result in a splitting of the resonance into three peaks with fixed ratios 1, 2, and 1. Interaction in a linear array of molecules at regular distances would produce five peaks with ratios 1, 4, 6, 4, and 1.)

FIG. 6. Relative susceptibility of tendon equilibrated at 66% R.H. as a function of rf field intensity, H_1. The drawn curve gives theoretical saturation behavior. O = outer peaks of the absorption at $\epsilon = 0°$; △ = middle peak at $\epsilon = 0°$; ● peak at $\epsilon = 55°$.

[19] F. Bloch, Phys. Rev. 70, 460 (1946).
[20] N. Bloembergen, Phys. Rev. 104, 1542 (1956).

The proton separation in a water molecule[21] is 1.59 Å. In the absence of motion the line splitting would amount to 21.0 gauss, when the magnetic field is directed along the vector $\mathbf{r}_{12}$, connecting the two protons, according to Pake's well-known formula.[22] In the case of rapid rotation or reorientation about a single axis, the splitting is reduced[23] by a factor

$$\tfrac{1}{2} \mid 3 \cos^2\zeta - 1 \mid,$$

where ζ is the angle between $\mathbf{r}_{12}$ and the reorientation axis. The splitting now is proportional to $\mid 3 \cos^2\epsilon' - 1 \mid$, where ϵ' is the angle between the field and the reorientation axis. If we assume that this narrowing process occurs, then the measurements indicate that: (a) the reorientation axis is directed along, or close to, the fiber axis; (b) $\zeta = 53°20' \pm 30'$ or $56°10' \pm 30'$, which is a requirement for obtaining a splitting of 0.75 ± 0.25 gauss, for $\epsilon' = 0°$. There is no angle of this magnitude related to the structure of the water molecule. The angle ζ is best approximated if the water molecules form chains in the fiber direction which rotate about the length axis of the chain, as shown in Fig. 7(a). With a tetrahedral structure of the water molecule, ζ equals 60°; with an H–O–H angle of 104.5°,[24] as in the gas state, ζ is slightly smaller.

It is worthy of note that some obvious structures of hydration can be ruled out or are unlikely:

(a) Water molecules bound to C=O or to available N—H groups with single hydrogen bonds and rotating about the bond axis are not compatible with the angular dependence, since these bonds are expected to be in the plane perpendicular to the fiber axis. If bonds close to the fiber direction exist, the angle ζ would be approximately 35° for the molecules bound to C=O, and 90° for the molecules bound to N—H.

(b) Motionless water molecules bound to specific sites, either by hydrogen bonds or polar forces, and having the direction of proton interaction along, or close to, the fiber direction could yield a splitting of decreased magnitude. The measured splitting would require that each molecule spend 5% of its time in such a site and 95% in a freely rotating state; the density in the gas phase, however, is many orders of magnitude too low to allow this distribution between gas and absorption sites. The possibility that the freely rotating molecules are adsorbed as well cannot be excluded, particularly since at 10% R.H. a narrow curve is observed; however, this seems unlikely. First, the freely rotating molecules are required to have a higher binding energy than the motionless molecules,

which is not to be expected. Second, one would expect, within limits, an increasing magnitude of the splitting with increasing relative humidity, since the population of the "motionless" sites would continually increase relative to the population of the "free rotation" sites.

(c) Freely rotating water molecules that translate in narrow channels between the collagen molecules would interact in the fiber direction, but they cannot yield the observed line shapes.

V. DISCUSSION OF WATER CHAINS

If water molecules form chains in, or close to, the fiber direction, another reorientation process can be considered that eliminates the need for an exact ζ or a perfect rotation, and yields results close to the observed values for the R.H. range 30%–80%.

From the work of Gränicher, Jaccard, and others[25] on the electric properties of single ice crystals it appears very likely that orientational defects of the Bjerrum type[26] (bonds that are unoccupied or doubly occupied by protons) migrate through the lattice by reorientation of the water molecules, in which process the protons pass through an angle of 109.5° but remain bound to the same molecule. Water molecules in a chain can have either of four configurations H, V, L, or S [see Fig. 7(b)], of which H and L are twofold degenerate. The conditions to be satisfied are the Bernal–Fowler rules[27]: one proton per bond and two protons per molecule. Simultaneous reorientations of two neighboring molecules, two examples of which are given in Fig. 7(b), are possible within the Bernal–Fowler rules, and could presumably occur at high rates. Proton jumps *along* hydrogen bonds are not probable because they either occur with the migration of ionic states that have a very low concentration, or produce ion pairs involving a high activation energy.

Each pair of reorientations will change the chain configuration, and the generation of configurations can best be considered as a Markov process. If we assume certain probabilities for each pair of reorientations, the probability of each configuration can be calculated. The average proton interaction can then be found. The possible proton positions are shown in Fig. 7(c), denoted by A–D. The center, O, represents the oxygen nucleus; OA and OB are in the H-bond directions. The lines 1–6 give possible directions of proton interactions. The probability of occurrence of interaction 1 will be denoted by p_1; of 2, by p_2; and of

[21] J. W. McGrath and A. A. Silvidi [J. Chem. Phys. **34**, 322 (1961)] find with NMR an average distance of 1.595 ± 0.003 Å for various crystal hydrates; the distances do not significantly depend on the type of hydrate.

[22] G. E. Pake, J. Chem. Phys. **16**, 327 (1948).

[23] H. S. Gutowsky and G. E. Pake, J. Chem. Phys. **18**, 162 (1950).

[24] N. Ockman and G. B. B. M. Sutherland, Proc. Roy. Soc. (London) **A247**, 434 (1958).

[25] H. Gränicher, C. Jaccard, P. Scherrer, and A. Steinemann, Discussions Faraday Soc. **23**, 50 (1957); H. Granicher, Z. Krist. **110**, 432 (1958); C. Jaccard, Helv. Phys. Acta **32**, 89 (1959).

[26] N. Bjerrum, Kgl. Danske Videnskab. Selskab. Mat.-fys. Medd. **27**, No. 1 (1951).

[27] These rules, from J. D. Bernal and R. H. Fowler, J. Chem. Phys. **1**, 515 (1933), lead for unspecified hydrogen positions to the half-hydrogen model for ice of L. Pauling, J. Am. Chem. Soc. **57**, 2680 (1935), which has been experimentally verified by neutron diffraction by S. W. Peterson and H. A. Levy, Acta Cryst. **10**, 70 (1957).

each of 3, 4, 5, and 6, by p_3. It can be shown that

$$[(3 \cos^2\theta - 1)]_{\text{Av}} = 3[p_1 - p_3 - (p_2 - p_3) \sin^2\phi] \cos^2\theta'$$
$$+ 3(p_2 - p_3) \sin^2\phi - p_1 - p_2 + 2p_3. \quad (7)$$

Here, θ is the angle between the field vector and the vector connecting two protons; θ', the angle between the chain axis [AB in Fig. 7(c)] and the magnetic field; and ϕ, the azimuth of the field vector with respect to the x axis. For rotation about the chain axis, $[\sin^2\phi]_{\text{Av}} = 1/2$, and (7) reduces to

$$(p_1 - \tfrac{1}{2}p_2 - \tfrac{1}{2}p_3)(3 \cos^2\theta' - 1). \quad (8)$$

The probabilities p_1, p_2, and p_3 have been calculated for chains of several lengths under the assumption of equal probabilities for all pairs of reorientations, except for the ones that start from an S configuration, from which either of two protons can jump and the probability of reorientation is taken to be doubled. For a chain length of four molecules the following values have been obtained:

$$p_1 = p_2 = 0.20711,$$

$$p_3 = 0.14645,$$

yielding the value 0.03033 for $p_1 - \tfrac{1}{2}p_2 - \tfrac{1}{2}p_3$. Thus the line splitting will be reduced to 0.64 gauss for $\theta' = 0°$, slightly lower than the observed value. Calculations have shown that the reduction factor has the same value (within 0.1%) for chains of all lengths from three molecules up.

The binding of water chains to the collagen molecules will change the probabilities p_i slightly, and since hydrogen bonds (both to nitrogen and to carbonyl-oxygen, participating in a chain) will increase the probability of the V configuration, the actual value is expected to be higher. Moreover, the crude model in these calculations does not justify a precise quantitative comparison.

It is observed from Eq. (7) that at $\theta' = 0$ the splitting does not depend on the azimuth ϕ, and hence is not affected by rotation of the chains. Rotation is required, however, to obtain the observed angular dependence.

The intermolecular interaction in a rigid chain would cause a significant broadening, which is not observed. It is therefore necessary to assume that the lifetime of a particular chain is short compared with the lifetime of a spin state. If chains are broken and rebuilt rapidly the second-neighbor interactions will be effectively reduced, but the reorientation process of each molecule will not be affected as long as the fraction of time spent by a molecule in a chain remains close to 1. The lifetime τ of a molecule in a chain can be estimated as

$$\tau \approx \nu^{-1} \exp(-E/RT), \quad (9)$$

where ν is the $O\cdots O$ stretching frequency of the hydrogen bond, which is approximately 6×10^{-12} sec^{-1}

(corresponding to 200 cm^{-1}),[28] and E is the activation energy required for the breaking of a chain, which can be estimated to involve two hydrogen bonds. Taking 5 kcal/mole for the energy of a hydrogen bond,[29] we obtain

$$\tau \approx 5.10^{-6} \text{ sec.}$$

The lifetime of a spin state is of the order of magnitude of 10^{-4} sec, as derived from the width of the absorption curves, hence the requirements for effective reduction of intermolecular interactions seem to be fulfilled.

The R.H. range for which line splitting is observed corresponds to an approximate plateau in the sorption isotherm of collagen.[30-31] The amount of absorbed water in this range, 20–30 g per 100 g dry collagen,[30] can form approximately 2.5–4 extended water chains per collagen threefold helical unit, whereas 2 channels are available per unit for close hexagonal packing of the collagen units. The narrow absorption curves obtained at low and high relative humidity can be explained as follows: At 10% R.H. the amount of water is insufficient for the formation of chains; each molecule is individually absorbed, apparently retaining a high degree of rotational freedom. At relative humidity above 80–90% the water molecules form structures in three dimensions, which may resemble the liquid structure, in which reorientation is less restricted than in the intermediate R.H. range.

The central absorption peak has to be attributed to water molecules whose reorientation is not restricted; possibly they occur in regions of macromolecules which do not favor the formation of restrictively reorientating water structures. Such regions may correspond to the collagen-band regions.[32]

VI. STRUCTURAL RELATION BETWEEN WATER AND COLLAGEN

Jacobson[4] discussed the structural relation between DNA and water and pointed out that the repeating distance along a DNA helix equals seven a-axis repeats of ice, extrapolated to room temperature. A similar relationship exists between collagen and water.

The proposed structure "collagen II"[33] is a threefold

[28] P. C. Cross, J. Burnham, and P. A. Leighton, J. Am. Chem. Soc. **59**, 1134 (1937). See also E. R. Lippincott and R. Schroeder, J. Chem. Phys. **23**, 1099 (1955), and C. G. Cannon, Spectrochim. Acta **10**, 341 (1958).

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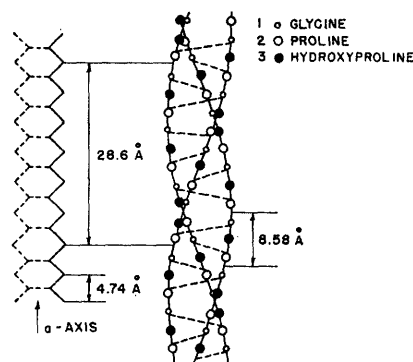
FIG. 8. Relation between collagen macromolecule, schematically represented at the right (after A. Rich), and water structures. Water chains are represented at the left; the dashed lines make an ice-I structure as seen along the c axis. Distances in the water lattice are values for ice extrapolated to 25°C.

helix, schematically represented in Fig. 8. The N—H of glycine in every third position is involved in intramolecular hydrogen bonding, but the C=O of glycine is available for hydrogen bonding to water, as is the case with the C=O of the residue in position 3 (the "hydroxyproline" position) and the N—H of some residues in positions 2 and 3. All of these groups have their bond axis roughly perpendicular to the axis of the helix. Other water binding positions are the charged groups and the hydroxyl groups of side chains. The repeating distance along the axis of the helix is 28.6 Å.

For the repeating distance along the a axis[34] of ice I at 0°C we may take 4.514 A, corresponding to a hydrogen-bond length of 2.76 A. Using 2.10^{-3} deg^{-1} for the coefficient of linear thermal expansion,[35] we find for the a axis repeat at 25°C, 4.74 A. This corresponds to a hydrogen-bond length of 2.9 Å, which is the value arrived at for water both by x-ray diffraction[36] and infrared measurements.[29] It now appears that the collagen repeat of 28.6 Å equals six a-axis repeats within an accuracy of 2 percent for the temperature range 17°–38°C. The a-axis repeat is nothing else but the second-neighbor distance in any water lattice that utilizes the tetrahedral angle, as in the aforementioned water chains. Thus *the collagen macromolecules may stabilize the existence of chainlike structures in the water of hydration by the formation of hydrogen bonds at appropriate sites.*

We have also investigated the possibility that the collagen molecule with its tenfold symmetry bears a relation to water structures in three dimensions. Indeed, it seems possible, from preliminary model

building,[37] that water can form cylindrically symmetrical structures, which have a tenfold symmetry resulting from the occurrence of pentagonal water structures perpendicular to the symmetry axis, and which are hexagonal along the axis.

If structural relations exist, it is likely that water is an important factor in stabilizing the intramolecular collagen type of configuration during cooling of gelatin solutions, as has been suggested recently by Harrington and von Hippel.[38] The particular model that these authors propose is not entirely satisfactory, both from a streochemical viewpoint[31] and because no evidence for this structure is found in the NMR spectrum. The x-ray data of Esipova et al.[39] are compatible with any semiregular arrangement of water molecules close to the macromolecules.

A single chain of water molecules may be bound to two collagen macromolecules; this would make water of major importance in intermolecular interactions.

VII. CONCLUSION

The NMR data indicate clearly that in the intermediate range of relative humidity (30%–80%) a large part of the water molecules in tendon reorient restrictively in such a way that the resulting proton interaction takes place in, or close to, the fiber direction. The experimental results are theoretically expected if the water molecules form chains (with tetrahedral angles between the bonds) in the fiber direction, which rotate or reorient about their length axis. Such chains will be extensively hydrogen-bonded to the collagen macromolecules that can make hydrogen bonds to water in the appropriate direction for chains to be formed, and that might even stabilize chains because of the existence of a perfect structural relationship. The lifetime of an individual chain is expected to be fairly short; the breaking and re-formation of hydrogen bonds in the chains and between macromolecules and water molecules provides the mechanism for chain rotation: A portion of a chain will become "loose" and attach to other sites of the same or of other macromolecules, thereby changing the angular position of the chain. At higher R.H. (above 80%–90%) enough water is present to form structures in three dimensions which may bear extensive structural relationships with the macromolecules, as suggested by preliminary model building. If such structures exist, however, they must have many lattice defects (as indicated by the comparably small width of the observed NMR ·

[34] This is the value used by Jacobson,[4] in agreement with the value used by Peterson and Levy.[27]

[35] Value used by Jacobson.[4] The correctness of this value is questionable, since other sources give lower values, but this does not influence the results greatly.

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resonance line at high relative humidity) and be in a state between the solid and liquid states.

Note added in proof: The narrow peak at 10% R.H. has not been confirmed by later measurements and may be due to the use of zinc chloride in the drying procedure.