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Published in:
Journal of Chemical Physics

DOI:
[10.1063/1.1679805](https://doi.org/10.1063/1.1679805)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1973

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Citation for published version (APA):
Migchelsen, C., & Berendsen, H. J. C. (1973). Proton exchange and molecular orientation of water in hydrated collagen fibers: An NMR study of H₂O and D₂O . *Journal of Chemical Physics*, 59(1), 296-305.
<https://doi.org/10.1063/1.1679805>

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Proton exchange and molecular orientation of water in hydrated collagen fibers. An NMR study of H₂O and D₂O

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Citation: *The Journal of Chemical Physics* **59**, 296 (1973); doi: 10.1063/1.1679805

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Proton exchange and molecular orientation of water in hydrated collagen fibers. An NMR study of H₂O and D₂O

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(Received 17 November 1971)

An experimental study of proton and deuteron magnetic resonance of the hydration of collagen as a function of water content, temperature, and the addition of salts has been made. From the temperature dependency of linewidths, correlation times for molecular rotation of water molecules and proton exchange times have been determined. For a water content of 45 g H₂O per 100 g collagen the rotational correlation time is 3×10^{-8} sec at 25 °C, with an activation energy of 4.8 kcal/mole, and the proton exchange time is 1.3×10^{-4} sec with an activation energy of 10 kcal/mole. Ammonium ions increase the proton exchange rate. The proton exchange most probably occurs between proton donating and proton accepting groups on the macromolecule, intermediated by hydrogen-bonded water molecules. It is shown that the exchange theory of Gutowsky, McCall, and Slichter is applicable to dipole coupling in anisotropic systems. From an analysis of the anisotropy of H₂O and D₂O rotation in terms of Saupe's parameters, it is concluded that a model with two specific water binding sites is consistent with experimental results.

I. INTRODUCTION

Due to the strong effect of molecular mobility on NMR linewidths it is possible to investigate the water protons in hydrated collagen separately from most of the macromolecular protons. The proton magnetic resonance (PMR) spectrum, disregarding a 10 G broad macromolecular resonance, of hydrated oriented collagen fibers consists of a nearly isotropic central line and an angular-dependent doublet caused by direct dipolar coupling in anisotropically rotating water molecules.¹⁻³ The separation of the doublet lines decreases with increasing water content. At increasing temperature the doublet merges into a single line.⁴ It was observed by us⁵ and later independently by Chapman and McLauchlan⁶ and by Dehl and Hoeve⁷ that the deuteron magnetic resonance (DMR) spectrum of D₂O-hydrated collagen shows a similar angular-dependent doublet, caused by quadrupolar interaction. No central line is observed as in the case of H₂O. The distance between the lines remains unchanged at increasing temperature and the conclusion was reached^{7,8} that the collapse of the proton doublet at increasing temperature is caused by proton exchange between water molecules. The anisotropic rotation of the water molecules can be described in terms of a tetrahedral model^{1,5} or, more generally, in terms of Saupe's orientation matrix $S^9,10$ as has been done by Chapman and McLauchlan⁶. Molecular models that will yield anisotropic rotations have been postulated on the basis of the existence of water chains^{1,6} and on the basis of defect diffusion in a solid-state structure.¹¹

In this work we describe more quantitative measurements on the collagen-water system, in which

the role of the proton exchange process is emphasized. From temperature-dependent studies the exchange times and the rotational correlation times of the water molecules have been found together with their activation energies at various water contents. The influence of salts on the proton exchange is described. The nature of the middle line is discussed.

II. EXPERIMENTAL METHODS

A. Sample Preparation

The collagen used in this study was rat-tail tendon. To obtain oriented samples the collagen fibers were wound parallel to each other on a thin Teflon disc, with dimensions of 7 mm × 8 mm × 0.6 mm for studies at room temperature, of 5 mm × 4 mm × 0.6 mm for PMR studies at variable temperatures, and of 5 mm × 6 mm × 0.6 mm for DMR studies at variable temperatures. The water contents were adjusted by equilibrating the samples in atmospheres with constant relative humidities, which were obtained in desiccators with a saturated solution of a suitable salt. For preparation of samples with the water completely or partly exchanged with D₂O molecules, the fibers were bathed for three hours in 99.9% D₂O or 50% D₂O, after which the D₂O was replaced by fresh D₂O with the same initial concentration. After allowing the fibers to stand overnight in the D₂O they were wound on Teflon discs and equilibrated at constant relative D₂O humidity. Water contents of the samples were determined from the loss of weight after drying the samples during three days above P₂O₅ at a temperature of about 85 °C.

B. NMR Measurements

NMR measurements were carried out with a Varian Wide Line NMR spectrometer with a field homogeneity of about 8 mG over 1 cm³ sample volume, operating at 60 MHz for PMR and at 10 MHz for DMR. The derivatives of the NMR absorption signals were obtained by field modulation with a frequency of 20 Hz. For variable temperature experiments Dewars were constructed with inner diameters of, respectively, 7 and 9.6 mm for the 60 MHz and 10 MHz probe. Spectra were recorded under nonsaturating slow passage conditions.

III. RESULTS

A. Influences of H₂O and D₂O Contents on NMR Spectra

The maximum values of PMR and DMR splittings in the spectra of collagen hydrated with, respectively, H₂O and D₂O, as observed when the fibers are oriented parallel to the applied magnetic field, have been determined as a function of the respective H₂O and D₂O content. In both cases a decrease of the doublet separation has been observed with increasing water content. The experimental results are summarized in Fig. 1. In this figure the splittings are expressed as reduction factors, while the absolute values of the observed splittings are denoted on the right-hand vertical scales. The reduction factor R_H for the PMR splitting is defined as the observed splitting relative to the value of 21.6 G as was found by Pake¹² for water molecules in crystals of CaSO₄·2H₂O and afterwards by others for various hydrates.¹³ The reduction factor R_D for the DMR splitting is defined as the observed quadrupole splitting relative to a value

of 530 G as expected for a fixed water molecule hydrogen bonded to other water molecules on the basis of an empirical equation given by Soda and Chiba.¹⁴ The proton splittings at the two highest water contents have been measured at reduced temperatures since the separation at room temperature decreases as a result of proton exchange processes that will be described in Sec. III D.

B. Interactions Contributing to the Linewidth

To get more information about the interactions contributing to the linewidth, PMR spectra of samples hydrated with H₂O and a 50% H₂O–50% D₂O mixture have been compared. In the case of hydration with a 50% H₂O–50% D₂O mixture the hydration water has the molecular composition: 25% H₂O, 50% HDO, and 25% D₂O. If intermolecular interactions contribute considerably to the linewidth, there should be a decrease in linewidth of the doublet lines compared with collagen hydrated with 100% H₂O. A reduction of about a factor of two is expected because the dipolar interaction between H and D nuclei is weak relative to the interaction between H nuclei. The doublet lines arise exclusively from H₂O molecules; thus the linewidth will not be influenced by partial replacement of H by D if intramolecular interaction is the main cause of line broadening. In Fig. 2 spectra (A) and (B) show the results at room temperature for hydrated collagen containing, respectively, 33 g 100% H₂O or a 50% H₂O–50% D₂O mixture per 100 g dry collagen. The doublet lines do not show significant differences in separation and linewidth. The separations are respectively 730 and 770 mG. The widths of the Lorentzian doublet lines are 120 and 108 mG. These differences are within the reproducibility of sample preparation. Hence it can be concluded that the principal contribution to the linewidth is given by intramolecular interactions. The broadened central line in spectrum (B) can be attributed to the superposition of the same narrow line as is visible in spectrum (A) and the unresolved triplet of the HDO molecules. As shown in Fig. 2, spectrum (C), the low temperature broadening is considerably larger for the doublet lines than for the central line. This also excludes intermolecular interaction as the main cause of broadening because in that case the H₂O and HDO lines should show a similar broadening.

C. Nature of the Central Line

To investigate whether the central line in the collagen–H₂O spectra results from isotropically rotating water molecules or from protons of other origin, the H₂O molecules have been exchanged with D₂O. The PMR spectrum of a sample containing 34.5 g D₂O per 100 g dry collagen consisted of one single line with a maximum peak-to-

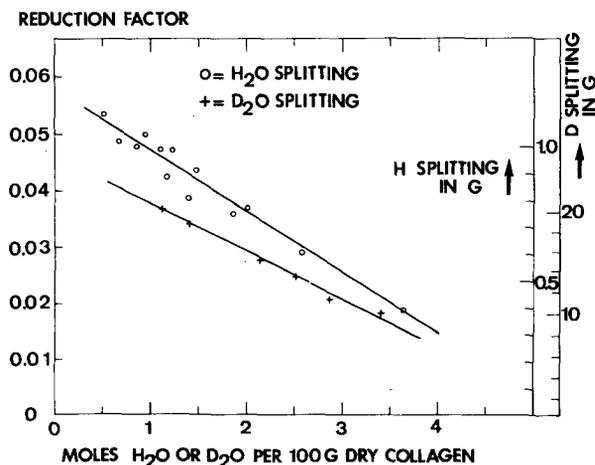


FIG. 1. Dipole and quadrupole splittings of water in oriented collagen with fiber-to-field angle of 0° as a function of water content. The two right scales give the splittings expressed in Gauss, while the left hand vertical scale gives the reduction factors.

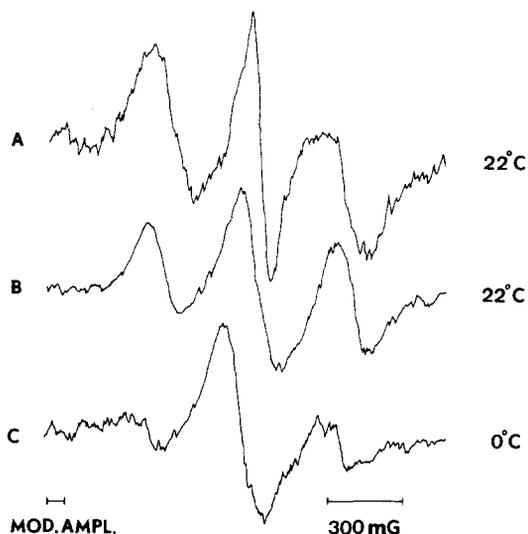


FIG. 2. Spectra of collagen hydrated with a 50% H_2O -50% D_2O mixture at temperatures of 22°C and 0°C (B and C) together with a spectrum of collagen hydrated with H_2O (A). The water content of the samples was 33 g per 100 g dry collagen. The fibers were oriented parallel to the magnetic field.

peak distance of 80 mG, for a fiber orientation parallel to the magnetic field. A minimum line width of 50 mG occurs when the angle between fibers and field is 55°. The linewidth corresponds with that observed for the central line of collagen- H_2O with the same water content. The intensities have not been compared quantitatively but seem not to differ considerably. An estimation of the intensity yields values between 1 and 2 g H per 100 g collagen. This result indicates that the line is not caused by water protons. Moreover, if the central line was supposed to be caused by isotropically rotating water molecules, the remaining line in collagen- D_2O should be due to a small amount of protons in the hydration water, which occur practically only in HDO molecules. In that case the line width, due to anisotropic dipolar H-D interaction should be 160 mG, which is twice the observed value. Thus the assumption made earlier that the central line is due to water protons has to be rejected. This conclusion is in agreement with that of Dehl and Hoeve,⁷ but not with that of Khanagov and Gabuda,³ who attribute the central line to capillary water on the bases of the agreement of temperature dependent behavior with that of capillary water in other systems. The middle line is probably mainly due to mobile side chains of the protein, although the presence of small molecules cannot be entirely excluded.

D. Temperature Dependence of H_2O and D_2O Spectra

The temperature dependence of the PMR spectra of collagen hydrated with 22, 28, 45, and 82.5 g

H_2O per 100 g collagen has been investigated. Spectra for a 45 g H_2O per 100 g collagen sample are shown in Fig. 3. The doublet lines show a minimum width of 67 mG in the temperature range from 2.5 to -12°C. The doublet separation is nearly constant (720 mG) below -12°C, while above 2.5°C the separation decreases and the doublet finally coalesces into a single line above 30°C. The central line has a constant width of 65 mG, but decreases in amplitude at decreasing temperature. The weak extra doublet observed in spectra D and E with a separation half that of the main doublet is an artefact due to the method of winding fibers on a disc, which causes a small fraction of the collagen fibers to be oriented at 90° with respect to the field. For samples with other water contents the influence of temperature is qualitatively the same, but quantitatively there are considerable differences. The temperature corresponding to the minimum linewidth and the temperature where the doublet changes into a single line, increase with decreasing water content.

For a sample with a water content approximately corresponding to the native state, no fine structure is observed at room temperature, as is shown in Fig. 4. Below 15°C this sample shows a doublet

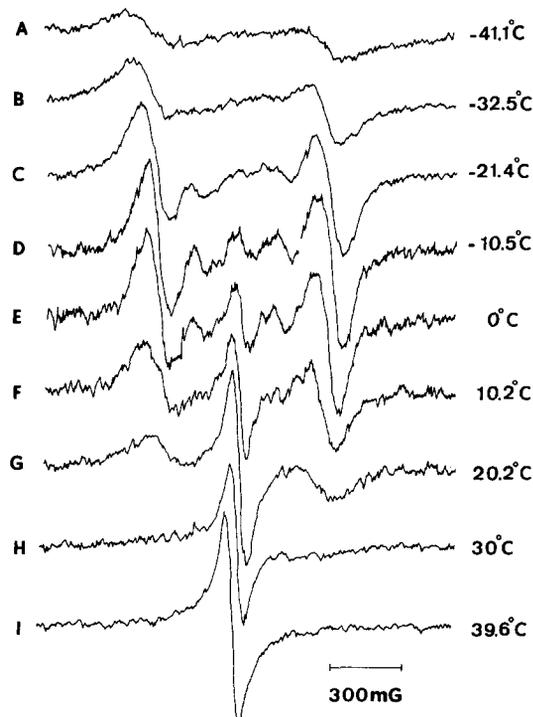


FIG. 3. PMR spectra of collagen with a water content of 45 g per 100 g dry collagen at different temperatures. Spectra H and I are reduced twice in amplitude relative to the other spectra, due to different instrumental adjustments. Fibers were oriented parallel to the magnetic field.

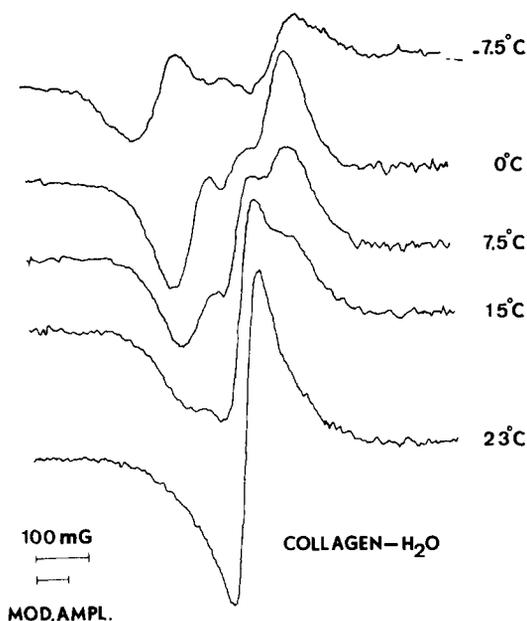


FIG. 4. PMR spectra of collagen with a water content of 82.5 g per 100 g dry collagen at different temperatures. Fibers were oriented parallel to the magnetic field.

structure such as is observed for partly dried samples at room temperature. A minimum width of the doublet lines of 56 mG is found in the temperature range from -7.5°C to 7.5°C . At a temperature of -7.5°C there is a well-resolved splitting of 230 mG between the outer lines. The central line is observable at temperatures above 7.5°C . For collagen hydrated with D_2O , the DMR doublet shows a broadening at lower temperature similar to that observed in PMR spectra of collagen- H_2O . At higher temperatures, however, no coalescence into a single line has been observed even with very high D_2O contents. In Fig. 5 some spectra are given

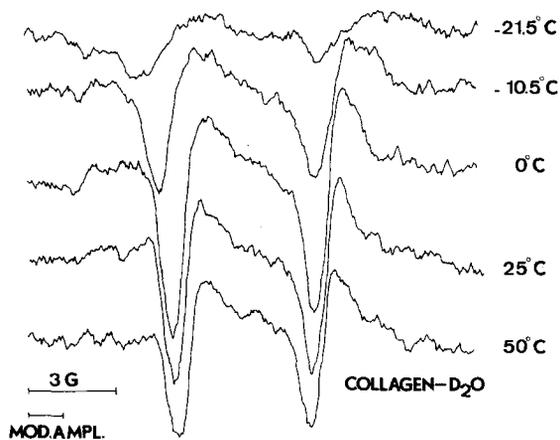


FIG. 5. DMR spectra at different temperatures of collagen hydrated with about 120 g D_2O per 100 g dry collagen and oriented parallel to the magnetic field.

for a sample with the high D_2O content of about 120 g D_2O per 100 g dry collagen. The doublet separation is slightly temperature dependent and amounts to 4.7 G at 25°C .

E. Influence of Salts

As has been shown,^{2,4} addition of salts containing proton donating or accepting ions to collagen influences the PMR spectra in the same way as raising the temperature. An explanation of this effect can be given by a proton exchange process.⁷ The results of a quantitative study on the effect of addition of NH_4Cl to collagen on the PMR spectra at room temperature are given in Fig. 6. An estimate of proton exchange times as a function of NH_4Cl concentration in the hydration water will be made in Sec. IV.

IV. INTERPRETATION OF TEMPERATURE AND SALT EFFECTS

The line broadening at lower temperatures in both PMR and DMR spectra can be explained by an increase in the rotational correlation time of water molecules. The line broadening and coalescence at higher temperatures of PMR spectra and the absence of this in DMR spectra can both be explained by proton exchange between different water molecules or between water molecules and proton

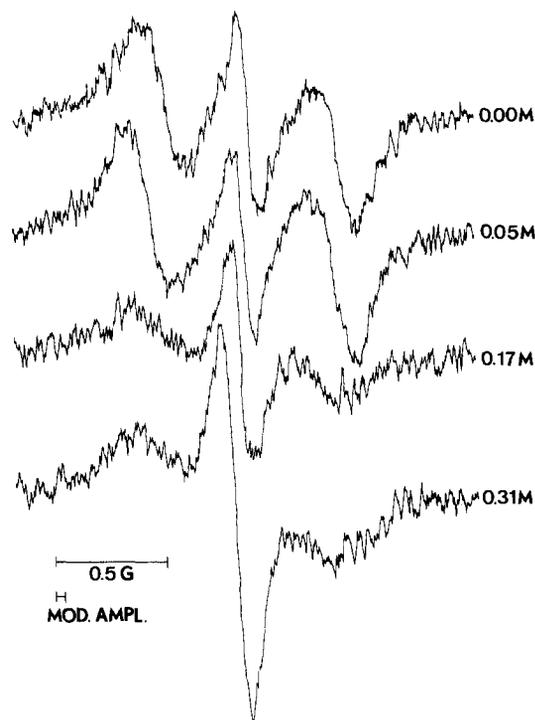


FIG. 6. PMR spectra of hydrated collagen with a water content of 31 g per 100 g dry collagen and different NH_4Cl concentrations in the hydration water. Fiber-to-field angle is 0° .

accepting or donating groups.^{7,8} Such processes will restrict the life time of a water molecule and thus influence intramolecular dipolar interactions that cause the doublet splitting in the PMR spectra. The DMR spectra, on the other hand, are determined by quadrupolar interactions that depend on electric field gradients at the sites of the deuterium nuclei. A jump of a deuteron to a neighboring water molecule results in a rotation of the field gradient over 180°, leaving the interaction unchanged.

To evaluate the correlation times and activation energies of the rotational and exchange processes, the PMR spectra have been analyzed quantitatively. Line shape analyses based on the nonoverlapping parts of the doublet lines show a Lorentzian shape of these lines. For samples with water contents of 22, 28, and 45 g H₂O per 100 g dry collagen, the linewidths $(\gamma T_2)^{-1}$ are plotted in Fig. 7 against the reciprocal absolute temperature. The linewidth observed before the lines merge is due to both intramolecular interaction characterized by a relaxation time $T_{2\text{rot}}$ and to the proton exchange process characterized by the exchange time τ , defined as the average lifetime of a water molecule. With this definition τ^{-1} represents the probability per second that one proton of a water molecule jumps. For the case of slow exchange a Lorentzian line shape is expected with a total linewidth characterized by

$$T_2^{-1} = T_{2\text{rot}}^{-1} + (2\tau)^{-1}. \quad (1)$$

This formula was derived by Piette and Anderson¹⁵ on the basis of the theory of Gutowsky, McCall, and Slichter¹⁶ that described the modification of the Bloch equations in the case of chemical exchange of atoms between two chemical species. This theory is not strictly applicable to the case

of exchanging protons with dipolar coupling. However, as is shown in the Appendix, a correct treatment based on Alexander's density matrix formalism, leads to an identical expression.

The plot of $\log T_2^{-1}$ versus the reciprocal absolute temperature can be decomposed into these two contributions. This is shown in Fig. 7, where the curved lines are obtained by summation of the two straight lines. This indicates that both processes have an exponential temperature dependence with a well defined activation energy for both. This way of analyzing spectra is only possible when the doublet structure is well resolved. The activation energies and τ_0 can be obtained by using the Arrhenius equations:

$$\tau = \tau_0 \exp(E_{\text{exch}}/RT), \quad (2)$$

$$T_{2\text{rot}}^{-1} = A \exp(E_{\text{rot}}/RT). \quad (3)$$

$T_{2\text{rot}}$ can be related with the rotational correlation time τ_c on the basis of the relaxation theory of Kubo and Tomita,¹⁷ which in the case of water with an interproton distance of 1.58 Å and a resonance frequency of 60 MHz yields

$$T_{2\text{rot}}^{-1} = 14.5 (3x + 5x/(1+x^2) + 2x/(1+4x^2)), \quad (4)$$

where $x = \omega_0 \tau_c$ and ω_0 the resonance frequency is in radians seconds⁻¹. This formula was used to derive τ_c from the corresponding $T_{2\text{rot}}$ values at 25°C. Unless $0.1 < \tau_c \omega_0 < 10$, $T_{2\text{rot}}^{-1}$ is approximately proportional to τ_c and E_{rot} can be used to determine the pre-exponential factor τ_0^{rot} in $\tau_c = \tau_0^{\text{rot}} \exp(E_{\text{rot}}/RT)$. For the values of τ_c obtained at 25°C the condition on τ_c is fulfilled. From Table I it appears that the values for τ_0^{rot} at different water contents agree within the estimated accuracy. At increasing water content, the activation energy of the rotational process decreases, resulting in an

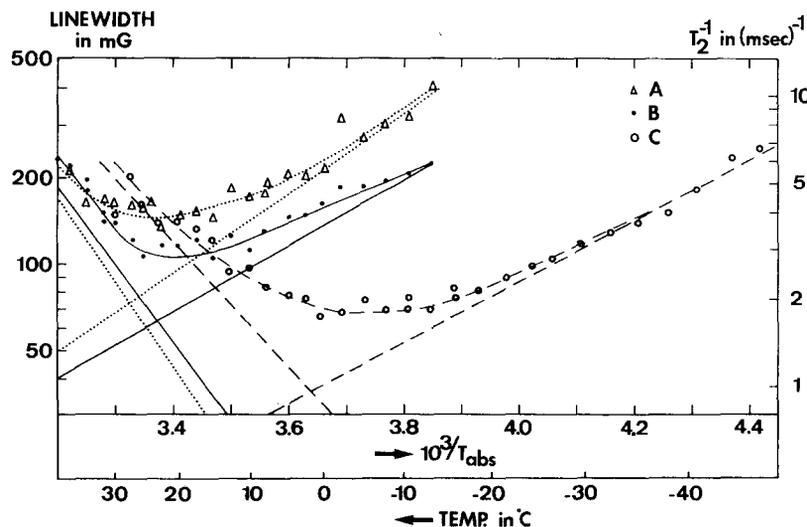


FIG. 7. Line width in milli-Gauss (left-hand vertical scale) and in (msec)⁻¹ (right) of hydrated collagen samples with water contents of respectively 22, 28, and 45 g H₂O per 100 g dry collagen (A), (B), and (C), plotted versus reciprocal absolute temperature. Curved lines are the sum of two exponentials, which result from rotational and exchange broadening.

TABLE I. Data on rotation and proton exchange obtained from analysis of PMR results on hydrated collagen with different water contents.

Water content per 100 gram collagen	22 ± 1 g (1.2 mole)	28 ± 1 g (1.55 mole)	45 ± 1 g (2.5 mole)
$T_{2,rot}$ (msec) at 25 °C	0.5 ± 0.1	0.6 ± 0.1	2.0 ± 0.3
τ_c (rotation) at 25 °C (sec)	(1.3 ± 0.3) · 10 ⁻⁷	(1.0 ± 0.2) · 10 ⁻⁷	(0.30 ± 0.05) · 10 ⁻⁷
τ_0^{rot} (sec) ^a	1.4 × 10 ⁻¹¹	1.6 × 10 ⁻¹¹	1.0 × 10 ⁻¹¹
activation energy for rotation (kcal/mole)	5.4 ± 0.2	5.2 ± 0.2	4.8 ± 0.2
τ (exchange) at 25 °C (sec)	(3.2 ± 0.6) · 10 ⁻⁴	(2.7 ± 0.5) · 10 ⁻⁴	(1.3 ± 0.3) · 10 ⁻⁴
τ_0 (exchange) (sec) ^b	1.3 × 10 ⁻¹³	3.7 × 10 ⁻¹³	8 × 10 ⁻¹²
activation energy for proton exchange (kcal/mole)	13.0 ± 0.4	12.3 ± 0.4	10.0 ± 0.4

^aThe τ_0^{rot} values have an accuracy of a factor of 1.6.

^bThe τ_0 (exchange) values have an accuracy of a factor of 2.

increase of observed $T_{2,rot}$ values at 25 °C. For the exchange process τ_0 increases with increasing water content. However, a decrease of τ at 25 °C is found with increasing water content due to a decreasing activation energy.

The effect of NH_4Cl on the hydration of collagen (Fig. 6) can be analyzed by comparing the linewidth of these spectra with the results obtained from the analysis of the temperature dependent behavior of a series of spectra of collagen without NH_4Cl but with about the same water content. The line splitting at low temperatures is not influenced by the presence of NH_4Cl . Since the linewidth of the doublet lines is given by Eq. (1), the proton exchange time can be derived from the measured linewidth T_2 if $T_{2,rot}^{-1}$ is known. If it is assumed that $T_{2,rot}^{-1}$ is not influenced by the added NH_4Cl , a value of 1.67 msec⁻¹ for this contribution to the linewidth is obtained from Table I. The difference in water content of the investigated sample (31 g per 100 g dry weight) and the sample for which the $T_{2,rot}^{-1}$ value of 1.67 msec⁻¹ has been derived (28 g per 100 g dry weight) will not be important as evidenced by the data of samples with different water contents in Table I. In Fig. 8, the obtained values for τ^{-1} , the probability per second that one proton of a water molecule exchanges, are plotted versus the NH_4Cl concentration. Since the points are lying approximately on a straight line, the relation between τ^{-1} and the NH_4Cl concentration can be expressed as

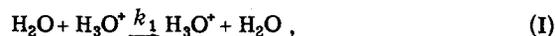
$$\tau^{-1} = -[\text{H}_2\text{O}]^{-1} (d[\text{H}_2\text{O}]/dt) = k[\text{NH}_4^+] + A, \quad (5)$$

where A represents the constant contribution to τ^{-1} caused by other ions or groups. From the slope of the straight line in Fig. 8, a value of $k = 1.7 \times 10^4$ l mole⁻¹ · sec⁻¹ has been derived. Comparison of the effect of lowering the temperature of a sample with a 0.15 M NH_4Cl concentration and a NH_4Cl -free sample showed a shift of the temperature where the minimum linewidth is observed from 15 °C down to 12.5 °C and a higher value for the minimum value of the linewidth. These results are in good agreement with the assumption that NH_4Cl only influences the proton exchange time τ .

V. DISCUSSION

A. Proton Exchange Process

To discuss the possible molecular nature of the proton exchange process, a comparison with similar processes in liquid water can be made. The process of proton exchange in normal water has been studied with PMR in water enriched with ¹⁷O by Meiboom¹⁸ and with ¹⁷O resonance by Glastel¹⁹ and by Rabideau and Hecht.²⁰ Meiboom obtained for the exchange time a value of 1.1×10^{-3} sec and proposed the reactions:



From the pH dependence of the proton linewidth values for k_1 and k_2 of respectively 10.6×10^9 and 3.8×10^9 l mole⁻¹ · sec⁻¹ were derived. For these reactions activation energies of 2.6 and 4.8 kcal/

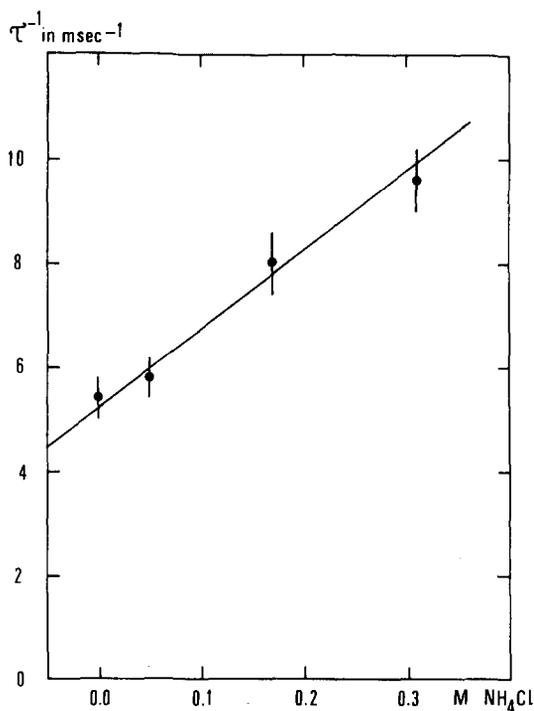
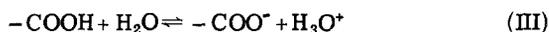


FIG. 8. Probability τ^{-1} per millisecond that one proton of a water molecule exchanges, as a function of the NH_4Cl concentration in the hydration water.

mole were found by Loewenstein and Szoke²¹ and of 2.4 and 2.1 kcal/mole by Luz and Meiboom.²² From a study of the effect of proton exchange on the ^{17}O linewidth values for k_1 and k_2 of 8.2×10^9 and 4.6×10^9 l mole⁻¹ · sec⁻¹ were found by Rabideau and Hecht.²⁰ The differences with the values as obtained by Meiboom¹⁸ are possibly caused by influence of quadrupolar relaxation in the ^{17}O study. It is also possible that in hydrated collagen the reactions I and II occur, as well as reactions like



and similar reactions for exchange on histidine and amino groups. From an investigation of all such reactions in which H_3O^+ or OH^- participate in the exchange, using known reaction constants,²³ it appears that reactions I, II, and III are dominating in the exchange rate. However, these reactions lead to pre-exponential factors of the order of 10^{-6} sec at pH 7, which exceed the observed values by several orders of magnitude. Another possibility is that the proton exchange occurs through chains of a few water molecules between fixed H-donating groups like $-\text{COOH}$, $-\text{NH}_3^+$, HisH^+ and H-accepting groups like $-\text{COO}^-$, $-\text{NH}_2$, and $-\text{His}$. Such processes are expected to have much smaller preexponential factors, which may approach a limiting value of $h/kT = 1.6 \times 10^{-13}$ sec when the average distance between donating and accepting

groups is small enough to allow the formation of water bridges.

At increasing water content the average number of water molecules between proton-exchanging groups of the protein is expected to increase. This will lead to a higher negative entropy of activation, because the probability that the intermediate water molecules have the correct orientation for proton transfer decreases. Hence the pre-exponential factor is expected to increase with increasing water content, in agreement with our experimental results.

Although the observed exchange times are of the same order as those observed in liquid water, the exchange mechanism appears to be quite different and bears more resemblance to the exchange between HPO_4^{2-} and H_2PO_4^- which occurs in aqueous solution via two water molecules.²⁴

B. Preferred Orientation of Water Molecules

To correlate the experimental reduction factors R_H and R_D with an anisotropic distribution of orientations of the water molecules, two ways of description can be used. In the first, chainlike water structures in which the H-H directions are oriented along one of the six sides of a regular tetrahedron are assumed in order to evaluate the probability distribution of orientations. Since the signs of the reduction factor are undetermined, four different distributions over the possible orientations are in agreement with the experiments. A more general description, without assumptions about the hydration structure, has been given by Chapman and McLauchlan⁶ in terms of three S parameters, as defined by Saupe⁹ for description of average ordering of molecules in nematic liquid crystals. In this description, the average orientation of a molecule with respect to a macroscopic axis of axial symmetry (orientation axis) is expressed as a tensor S , the elements of which are given by

$$S_{ij} = \frac{3}{2} \overline{\cos\theta_i \cos\theta_j} - \frac{1}{2} \delta_{ij}, \quad (6)$$

where θ is the angle between the i th coordinate axis of the molecule and the orientation axis, which is lying along the fiber direction.

When S is not zero, anisotropic interactions of tensorial character, as dipole or quadrupole interactions, do not average out to zero. The remaining average interaction yields a splitting which depends on the angle θ between orientation axis and magnetic field according to $(3\cos^2\theta - 1)$. The average interaction T , measured as the dipole or quadrupole splitting in the direction of the orientation axis, is given by

$$T = \frac{2}{3} \text{Tr}(\mathbf{S} \cdot \mathbf{T}), \quad (7)$$

where \mathbf{T} is the dipole or quadrupole interaction tensor on the molecular axes.

With the same convention for the molecular axes as was used by Chapman and McLauchlan⁶ (1 is bisector of the HOH angle, 2 in the H₂O plane along the interproton vector, 3 perpendicular to the H₂O plane) the reduction factors R_H and R_D can be related to the S parameters:

$$R_H = S_{22}, \quad (8)$$

$$R_D = (0.433 S_{11} + 0.667 S_{22}). \quad (9)$$

For the latter values an angle of 52° between molecular axis 1 and the principal axis of the field gradients at both deuterium nuclei and an asymmetry parameter η of 0.1 for the field gradient has been assumed. Implicit in the derivation of the S parameters from R_H and R_D is the assumption that the average orientations of H₂O and D₂O are equal and that R_D is not influenced by deuteron exchange with macromolecular groups with a fixed orientation. Due to the undetermined signs of the reduction factors, this way of description also leads to four possibilities. The four sets of S parameters for a water content of 2 moles per 100 g dry collagen, are given in Table II. The absolute values for S_{11} and S_{22} are in good agreement with the results obtained by Chapman and McLauchlan⁶ for collagen containing about the same amount of hydration water. However, they have obtained the sign of S_{11} incorrectly in the first and fourth case, resulting in different S_{33} values since S_{33} is found from the condition $\text{Tr}(\mathbf{S}) = 0$.

Chapman and McLauchlan have tried to select one of the four sets of S parameters as the correct one by measurement of the dielectric anisotropy at 40 MHz. They selected the S set given in the third row of Table II. However, their experimental values $\epsilon_{11} = 9.89$ and $\epsilon_{\perp} = 8.07$ for wet collagen can be fully explained by macroscopic anisotropy in sample structure, so that these experiments do not give a basis for selection between the four sets of S parameters.

TABLE II. Saupe's order parameters (S) for different sign combinations of the reduction factors R_H and R_D for proton dipole and deuteron quadrupole splitting. Sample: collagen containing 2 mole of H₂O per 100 g collagen at 25 °C.

R_H	R_D	S_{11}	S_{22}	S_{33}
+0.036	+0.029	+0.012	+0.036	-0.048
+0.036	-0.029	-0.122	+0.036	+0.086
-0.036	+0.029	+0.122	-0.036	-0.086
-0.036	-0.029	-0.012	-0.036	+0.048

Another method that can give additional information about the S parameters is the dichroism of the water absorption band at 5150 cm⁻¹. Fraser and Macrae²⁵ have observed a perpendicular dichroism for this band in kangaroo-tail tendon equilibrated with the laboratory atmosphere. The absorption band has been assigned to a combination of the antisymmetrical stretching and symmetrical deformation modes of the water molecule so that the associated transition moment is parallel to the line joining the two hydrogen atoms. They concluded that the water molecules were bound to the C=O groups projecting radially outwards from the collagen molecules. An orientation of water molecules perpendicular to the macromolecular axis was suggested. From their results a value of -0.1 for S_{22} can be derived. This result is, as far as the sign is concerned, consistent with the third and fourth set of S parameters in Table II. The absolute value, however, exceeds the NMR values by a factor of 3, which can be partly attributed to a lower water content in the optical experiments. Another possibility is that very strongly bound water molecules with a well defined orientation are not observed in the NMR spectra.

On the basis of correlation between intensity changes of the wide-angle x-ray pattern with hydration, Esipova *et al.*²⁶ have proposed that the crystalline regions might be stabilized by double hydrogen-bonded water bridges to the oxygen atoms of the peptide carbonyl groups. A theoretical investigation of the interchain bonding between the three peptide chains in the triple-helical collagen structure has been performed by Ramachandran and Chandrasekharan.²⁷ They have explored different possibilities in detail by use of computer methods and selected a structure in which the peptide chains are connected by one direct hydrogen bond between an -NH and a C=O group for every three residues, while a second hydrogen bond is made through a water molecule. One more set of interchain linkages can be formed by hydrogen bonding of a second water molecule to two carbonyl oxygens, as had also been concluded from x-ray experiments.²⁶ From this proposed structure the S parameters of the interchain water molecules can be estimated. For the water molecules bound between -NH and C=O groups the values $S_{11} = +0.12$, $S_{22} = +0.085$, and $S_{33} = -0.205$ are found, while the nearly horizontally positioned water molecules between two carbonyl oxygen atoms give approximately $S_{11} = S_{22} = -0.5$ and $S_{33} = 1$. If, for comparison with our experimental results on collagen with 2 moles H₂O per 100 g dry weight, we assume besides (2/3) mole of oriented water (4/3) mole to be present without preferred orientation, the S values will be $S_{11} = -0.06$,

$S_{22} = -0.07$, and $S_{33} = +0.13$. These S parameters correspond in sign and in approximate relative magnitude to the fourth set in Table II.

Summarizing it appears that only the third and fourth set of S values of Table II are in agreement with infrared data, while a model in which the anisotropy is caused by specific binding of water to the macromolecules is consistent with the fourth set. Physically this means a preferential orientation of water molecules with their H-H direction perpendicular to the fiber axis. Thus, a chain model of hydration can no longer be supported as the only possible or even the most likely model. A specific binding model (which was also supported by Fung and Trautmann²⁸) also reasonably explains the extremely long rotational correlation times found from the line broadening. In the case of specific binding these correlation times will be closely related to the lifetime of a water molecule in a bound position.

ACKNOWLEDGMENTS

We are indebted to J. J. de Vries and C. Dijkema, who cooperated with us in various stages of this research.

APPENDIX: THE EFFECT OF EXCHANGE ON DIRECT DIPOLE COUPLING

The GMS equations of Gutowsky, McCall, and Slichter,¹⁶ often used to calculate the effect of exchange on magnetic resonance line shapes, are based on an intuitive extension of the Bloch equations. From quantum-mechanical treatments, based on the equations of motion of the density matrix,²⁹⁻³¹ it is known that the GMS equations are not correct if isotropic spin-spin interactions occur which are not small with respect to the chemical shift difference. Moreover, the GMS results are incorrect by a factor of 2 for the limit of fast exchange rates. It appears that the case of chemical exchange in anisotropic systems that show line separation due to direct dipolar coupling has not been treated in the literature. For water in hydrated oriented biopolymers, as well as for nematic liquid crystals, a quantum-mechanical treatment of exchange broadening of dipolar splitting is required. In the following we shall show that for a system of molecules with two equivalent protons with nonvanishing dipolar interaction the GMS formula gives, except for a factor of 2 in the exchange time, an exact description of the effect of proton exchange between different molecules.

We shall use the formalism of Alexander, who gives an expression [Eq. (21) of Ref. 31] for the equation of motion of the density matrix (in the rotating frame) of a coupled system of protons, some of which are exchanging with protons on

equivalent but uncorrelated systems;

$$\begin{aligned} d\langle k\alpha | \rho | k'\alpha' \rangle / dt = & [\delta(k, k') \bar{\rho}^{\alpha\alpha'} + \delta(\alpha, \alpha') \bar{\rho}^{kk'} \\ & - \langle k\alpha | \rho | k'\alpha' \rangle] / \tau \\ & + i\langle k\alpha | [\rho, \mathcal{H}] | k'\alpha' \rangle. \end{aligned} \quad (\text{A1})$$

Here k, k' number the quantum states of exchanging protons and those of nonexchanging protons. The average life time between two exchanges on one molecule is τ . The $\bar{\rho}$ are averages over the exchanging and nonexchanging subspaces:

$$\bar{\rho}^{\alpha\alpha'} = \sum_k \langle k\alpha | \rho | k\alpha' \rangle / m, \quad (\text{A2})$$

m = dimension of exchanging subspace,

$$\bar{\rho}^{kk'} = \sum_\alpha \langle k\alpha | \rho | k'\alpha \rangle / n, \quad (\text{A3})$$

n = dimension of nonexchanging subspace.

The Hamiltonian in the rotating frame for two dipole-coupled protons is (in units of angular frequency)

$$\mathcal{H} = (\omega_0 - \omega)(I_z^1 + I_z^2) - \omega_1(I_x^1 + I_x^2) + 2\mathbf{I}^1\mathbf{D}\mathbf{I}^2, \quad (\text{A4})$$

where ω_0 is the Larmor frequency, ω the frequency and $\omega_1 = \gamma H_1$ the frequency of the driving rf field, and \mathbf{D} the dipole coupling tensor.³² In the case of an axially symmetric system in which fast averaging occurs around the axis of symmetry, as is true in the cases of interest, and with the magnetic field in the direction of the symmetry axis (z direction), the dipole tensor \mathbf{D} takes the following form:

$$\mathbf{D} = D \begin{pmatrix} -\frac{1}{2} & 0 & 0 \\ 0 & -\frac{1}{2} & 0 \\ 0 & 0 & 1 \end{pmatrix}, \quad D = \frac{2}{3} \text{Tr}(\mathbf{D}^m \cdot \mathbf{S}), \quad (\text{A5})$$

where \mathbf{D}^m is the dipolar coupling tensor in the molecular axis system with principal value $\gamma^2 \hbar r^{-3}$ and \mathbf{S} is the Saupe order matrix.³³ The dipole term in Eq. (A4) can now be written as

$$2D[I_z^1 I_z^2 - \frac{1}{2}(I_x^1 I_x^2 + I_y^1 I_y^2)]. \quad (\text{A6})$$

If we number the two-proton quantum states, for which we use the product wavefunctions of the protons in I_z presentation as a basis set, 1 to 4 for the (k, α) combinations (1, 1), (1, 2), (2, 1), (2, 2), respectively, Eq. (A1) yields the following equations of motions for those components of the density matrix that are needed to determine the resonance line shape $\text{Tr}[\rho \cdot (I_x^1 + I_x^2)]$.

$$\dot{\rho}_{12} = (\rho_{34} - \rho_{12}) / 2\tau + i(\Delta - D)\rho_{12} - \frac{1}{2}iD\rho_{13} + \frac{1}{2}iC - \rho_{12} / T_2 \quad (\text{A7a})$$

$$\dot{\rho}_{13} = (\rho_{24} - \rho_{13}) / 2\tau + i(\Delta - D)\rho_{13} - \frac{1}{2}iD\rho_{12} + \frac{1}{2}iC - \rho_{13} / T_2 \quad (\text{A7b})$$

$$\dot{\rho}_{24} = (\rho_{13} - \rho_{24})/2\tau + i(\Delta + D)\rho_{24} + \frac{1}{2}iD\rho_{34} + \frac{1}{2}iC - \rho_{24}/T_2 \quad (\text{A7c})$$

$$\dot{\rho}_{34} = (\rho_{12} - \rho_{34})/2\tau + i(\Delta + D)\rho_{34} + \frac{1}{2}iD\rho_{24} + \frac{1}{2}iC - \rho_{34}/T_2 \quad (\text{A7d})$$

Here $\Delta = \omega - \omega_0$ and $C = \omega_1(\rho_{22} - \rho_{11})$. The high temperature limit (no saturation) has been applied by neglecting terms of higher order in C . A relaxation term with T_2 has been added. If we substitute

$$G_1 = \rho_{12} + \rho_{13} \quad (\text{A8a})$$

$$G_2 = \rho_{24} + \rho_{34} \quad (\text{A8b})$$

we obtain

$$\dot{G}_1 = (G_2 - G_1)/2\tau + i(\Delta - \frac{3}{2}D)G_1 + iC - G_1/T_2 \quad (\text{A9a})$$

$$\dot{G}_2 = (G_1 - G_2)/2\tau + i(\Delta + \frac{3}{2}D)G_2 + iC - G_2/T_2 \quad (\text{A9b})$$

We note that these equations are equivalent to the modified Bloch equations for small driving field, which yield the GMS equation. However, the exchange time that should be used in the GMS equation is *twice* the proton exchange time. In the slow exchange limit G_1 and G_2 represent two lines with a separation $3D$, which exceeds as is well known, the classical value by a factor of $(3/2)$.

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