Collagen: Mobile Water Content of Frozen Fibers

Abstract. *From the change in the nuclear magnetic resonance splitting of heavy water in oriented wet collagen fibers at low temperatures, it is estimated that about 0.6 gram of heavy water per gram of collagen does not freeze. This estimate has been confirmed by a measurement of the heat evolved on melting "frozen" wet collagen. The water that does not freeze retains liquid-like mobility even at temperatures as low as −50°C.*

The water of hydration in the fibrous protein collagen has been shown by nuclear magnetic resonance (NMR) spectroscopy (*1*, *2*) to be in a state of high mobility at temperatures well below the freezing point of ordinary water. Berendsen and Migchelsen have shown (*1*) that, even when the moisture content is moderately high (0.45 g of H_2O per gram of collagen), the water remains unfrozen at temperatures as low as −30°C. However, very highly hydrated collagen such as that found in living tissue (> 0.8 g of H_2O per gram of collagen) has apparently not been studied at low temperature by NMR spectroscopy. Both NMR and calorimetric examination of collagen fibers containing more than 0.5 g of H_2O per gram of collagen have led to the conclusion that water that is present in excess of this amount can be frozen. The unfrozen water retains its mobility at very low temperatures, in the presence of the ice phase.

All broad-line NMR spectra of D_2O were examined at 9.2 Mhz, with single scans of the magnetic field. The spectrometer used in this laboratory and typical spectra of water in collagen derived from animal tail tendon have been described previously (*2*). A liquid nitrogen boiler provided the cooling gas for the NMR samples, and the temperature was measured with a thermocouple mounted near the sample. The heats of melting were measured with a commercially available differential scanning calorimeter.

Heavy water rather than H_2O was used in the present work because, as shown previously (*2*), the NMR spectrum of D_2O is not subject to certain line-broadening and interference effects which tend to obscure the spectrum of H_2O in collagen (*3*). The NMR spectrum of D_2O adsorbed in a bundle of parallel tendon fibers (*4*) consists of a pair of lines separated by a factor $K(3\cos^2\theta - 1)$, where K is an empirical constant which decreases with increasing D_2O content and θ is the angle between the fiber and magnetic-field axes.

Broad-line NMR spectra of D_2O in parallel kangaroo tail tendon fibers were recorded as the samples were cooled from room temperature. In Fig. 1 the splitting constant K is plotted against temperature for one sample (A) which did not freeze over the observable temperature range (25° to −30°C), and for three more highly hydrated samples (B, C, and D) in which freezing occurred. Freezing was accompanied by an increase in K and by a decrease in the intensity of the NMR lines, due to ice formation. [Since the NMR spectrum of D_2O ice is very broad (*5*), it is not observed in the narrow scans used to record these spectra.] The decrease in intensity of the NMR lines below the freezing temperature was not great, an indication that a high percentage of mobile water remained in the "frozen" collagen fibers. There was some hysteresis between the cooling and warming curves, for samples B, C, and D, apparently due to supercooling of the water in the microcapillaries. No change was observed in the splitting on warming the samples up to about −10°C, where melting caused the splittings and line intensities to return rapidly to their original values for the unfrozen collagen.

Table 1. Calorimetric analysis of unfrozen water in frozen collagen fibers (in grams of H_2O per gram of collagen).

Total water content	Unfrozen water content*
0.623	0.520
0.722	0.516
0.744	0.485
0.887	0.529
1.004	0.537
1.394	0.531
	0.520 (av.)

* Difference between total and frozen water (see text).

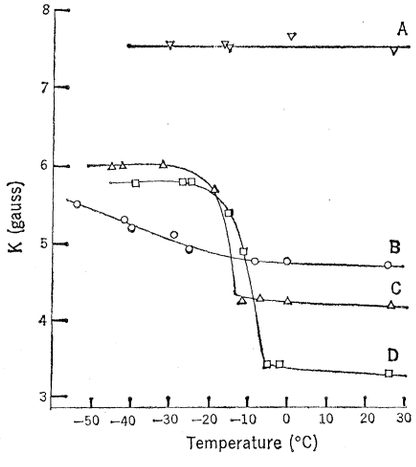

Fig. 1. Nuclear magnetic resonance splitting constant K of D_2O as a function of temperature for collagen fibers containing the following weight fractions of D_2O, based on the dry weight of the sample: (A) 0.38; (B) 0.70; (C) 0.825; (D) 1.10. In each case, the points were obtained in sequence, from higher to lower temperature.

It is interesting to try to estimate the quantity of mobile water remaining in the collagen at low temperature. Kuntz *et al.* (*6*) have estimated the amount of mobile water in frozen solution of biopolymers by direct integration of the absorption signals obtained from a high-resolution NMR spectrometer. However, in order to observe the mobile water in frozen collagen, it is necessary to use the broad-line NMR technique, in which the line intensities are dependent on several factors other than the relative numbers of nuclei involved. Nevertheless, the mobile water content can be estimated from the NMR splittings in samples C and D at low temperature, if one assumes the same relationship between K and D_2O content as that which holds at room temperature (*7*). (Sample B had very little "freezable" water in it and was apparently subject to severe supercooling, so that its largest observable splitting was probably not an equilibrium value.) The low-temperature K values of samples C and D are both about 6 gauss (1 gauss = 10^{-4} tesla), corresponding to a total moisture content at room temperature of slightly less than 0.6 g of D_2O per gram of dry collagen. This estimate is based upon the assumption that the K value of the D_2O is not significantly altered by the presence of ice in the fibers.

An independent estimate of the relative quantities of frozen and unfrozen water in the collagen was obtained by measuring the heat evolved on melting the frozen samples. Weighed samples of dried collagen were soaked in water, reweighed, and sealed in aluminum pans to prevent evaporation loss during the measurement. Each sample was frozen in the differential scanning calorimeter at −50°C, and the temperature was scanned through the

melting range. The melt endotherms of wet collagen obtained in this way were more diffuse than those of pure water, with the onset of melting commencing at least 15°C below the melting point of pure water. This is consistent with the freezing-point lowering expected for a liquid trapped in small, somewhat irregular capillaries. For each of six samples (Table 1) I calculated the quantity of ice from the measured heat of melting, using the value 79.71 cal/g (1 cal = 4.1840 joules) for the heat of fusion. As shown in the second column of Table 1, the amount of water remaining unfrozen was approximately the same for the six samples. Replicate endotherms on the same sample agreed to within ± 2 percent. However, some errors are necessarily involved in the preparation and weighing of the samples, and it is not likely that significant differences exist in this set of numbers.

The NMR estimate of the mobile water in frozen collagen, expressed in terms of H_2O rather than D_2O, is about 0.54 g of H_2O per gram of collagen. This amount is very close to the quantity obtained by calorimetry and implies that the NMR splitting constant K depends only on the amount of mobile water in the collagen and is not affected by the presence or absence of ice. It is also apparent from the combined results that essentially all of the water that does not actually freeze remains in a state of high mobility. On the basis of an average amino acid molecular weight of 90 (*8*), the "unfreezable" water amounts to 2.6 molecules per residue.

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References and Notes

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7. See (*4*), p. 764. A similar plot was obtained in this laboratory, extending over a wider range of moisture contents, from 0.2 to 1.2 g of D_2O per gram of collagen.
8. See, for example, J. E. Eastoe, in *Treatise on Collagen*, G. N. Ramachandran, Ed. (Academic Press, New York, 1967), vol. 1, chap. 1.
9. Supported in part by the U.S. Army Medical Research and Development Command, MOD No. 9914.

29 July 1970

Pheromone Transport and Reception in an Amphipod

Abstract. *Sexual dimorphism in the second antennae of the amphipod* Gammarus duebeni *Lilljeborg is connected with the reception in the male of a female sex pheromone transported through the water. Investigations on tritium-labeled specimens were carried out with scintillator and autoradiographic techniques.*

Experiments were designed to test whether the sexual dimorphism in the supposed chemo-sensory antennal receptor mechanisms in the amphipod *Gammarus duebeni* Lilljeborg indicates that the calceoli, not present in the female, are receptors for a female sex pheromone.

Females of *G. duebeni* were fed liver from rainbow trout which had received an intraperitoneal injection of [^{3}H]-acetylglucosamine. This diet produced radioactive accumulation in the females, as shown by liquid scintillation tests on whole animals (7,000 to 30,000 count/min) (Packard Tri-Carb liquid scintillation spectrometer, model 3320).

Unlabeled males were put into one compartment of an aquarium partitioned by a wall of double nylon net which permitted water circulation but prevented direct contact between the two compartments. When the labeled females were put into the other compartment, the males became agitated, and most of them swam to the net wall and clung to it. After some time the males again dispersed, which might indicate that the original gradient in the stimulus concentration had been obliterated by the circulation maintained in the aquarium. Control tests with males instead of females produced no activity.

After the males were exposed to the female stimulus for 30 to 60 minutes, they were removed and one group was prepared for liquid scintillation tests. The remaining specimens were fixed and prepared for light microscope autoradiography. Liquid scintillation measurements on the males were conducted on whole animals, on the second antennae (which carry the calceoli), and on the body minus the antennae (Table 1). The average weight of both second antennae of three males was 0.07 mg; the average weight of three whole animals was 33.70 mg.

The scintillation measurements indicated that the radioactivity per unit weight was approximately 1000 times higher in the second antennae than in the remainder of the body. Considering, however, the minute quantities of tissue and the limited number of sexually active males which could be used, the readings, which in the antennae were only 8 to 52 count/min above the background, could not be statistically significant. Nevertheless they contributed to focus the interest on the second antennae. Experiments from both the May and the November reproduction periods gave similar results.

Light microscope autoradiography was carried out on males in transverse

Table 1. Incorporation of radioactivity in males of *Gammarus duebeni*, kept for either 30 or 60 minutes in the same aquarium with labeled females of the same species. By means of a double, fine-meshed nylon net, direct contact between males and females was prevented, but there was free circulation of water between the two compartments. The figures represent counts, recorded for extracts of the animals measured for 20 minutes in a liquid scintillation spectrometer, and have been corrected for controls.

Subjects	Radioactivity		
	Second antennae	Total body minus second antennae	Total body
	Males kept for 30 minutes		
A	8	0	8
B	13	23	36
C	18	32	50
D	*	*	41
E	*	*	151
	Males kept for 60 minutes		
F	32	0	32
G	52	4	56
H	0	80	80
I	*	*	28
J	*	*	76
K	*	*	100

* Not measured.

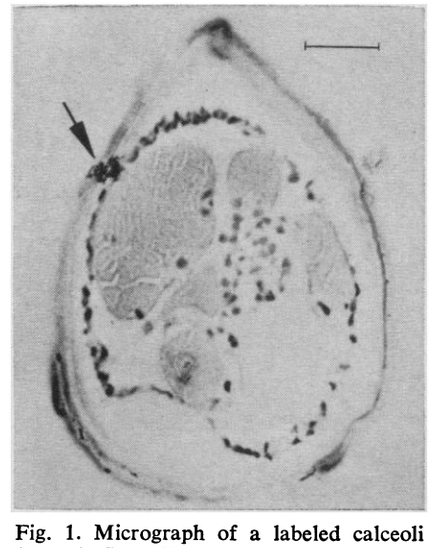

Fig. 1. Micrograph of a labeled calceoli (arrow). Bar, 50 μm.