*MODIFICATION OF THE STRUCTURE OF WATER IN AGAR GELS**

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The possibility that the structure of water is modified in the neighborhood of large structures with hydrogen-bonding surfaces to give rise to ordered lattices has been extensively discussed (cf. Bernal[1]) and considered in relation to biological systems.[2–5] Nuclear magnetic resonance (NMR) affords a technique to examine the possibility that "ordered" states of water, intermediate in rigidity of structure between "free" water and ice actually exist. Evidence from proton magnetic resonance studies indicates that the resonance signal of the water protons in systems containing macromolecules like deoxyribonucleic acid (DNA)[2] or tobacco mosaic virus (TMV)[6] as well as in certain cellular preparations (e.g., vaginal cell sediments and erythrocytes[7]) differ from those obtained with free water. It is not known, however, whether these changes are due to "ordered" water lattices or to other factors which influence the NMR signal. Thus, the reported broadening and decreased area of the proton signal of the water in systems of DNA[2] and TMV,[6] interpreted as evidence for "ordered water lattices," are regarded by Balazs, Bothner-By, and Gergely[8] as the influence of diamagnetic anisotropy in molecules of this type upon the proton signal, rather than resulting from "structuring" of water.

We wish to report high resolution NMR studies on agar gels which at present can best be interpreted as indicating that the water in such a gel is in a modified "state" with properties of structural rigidity intermediate between ice and free water. Other factors which might give rise to the NMR results have been systematically examined and found not to be responsible for the altered magnetic resonance properties of water in the agar gel system.

Materials and Methods.—The NMR spectra were obtained using a Varian Model V4302 high resolution NMR spectrometer, operated at a frequency of 60 Mc/s. The field was swept at 1.4 cps/sec unless otherwise stated. The areas of the NMR signals were obtained by weighing traces of the signal and comparing these to the weights of the traces of NMR signals from known H_2O standards, prepared by diluting H_2O with D_2O. T_1 for the water signal, when measured, was obtained using the direct method.[9] The line width of the proton signal for water was measured at the half-height of the peak amplitude. The charts were calibrated by using side-bands produced by a Hewlett Packard wide-range oscillator (Model 200 CDR). The samples were placed in calibrated 5 mm. O.D. tubes and measurements were carried out at room temperature (30°C) unless otherwise specified.

The agar employed was Bacto-agar (Difco); similar NMR results were obtained after the sample was exhaustively leached with water, ethanol, acetone, and ether. Two USP samples of gelatin (Difco and Knox) were studied and gave similar results. Samples of methoxycellulose (Methocel, Dow Chemical Co.) of varying viscosity grades (15, 400, 1,500, 4,000, and 8,000 centipoises) and of sodium carboxymethyl cellulose (Hercules Powder Co., CMC-7HCP, CMC-12HP, CMC-7MP,

and CMC-7LP) were used. Polyvinyl pyrollidone (PVP-30, General Aniline & Film Corp.) and pectin (Nutritional Biochem. Corp.) were also studied.

Results.—When examined under high resolution NMR spectroscopy, the line width of pure water measured under our conditions is 1.6 ± 0.4 cps, and this value is not significantly altered when water is progressively diluted with D_2O. In marked contrast, water protons of agar gels at room temperature give a much broader signal with decreased amplitude relative to the water standard. The line widths vary from about 5 cps for a 1 per cent agar to about 50 cps for a 10 per cent gel. With 3 per cent agar gels, the concentration used for most of these studies, the line width is about 16.5 cps, with a variation of about ±3 cps. The area under the curve of H_2O–D_2O mixtures (ranging from 100%–25% H_2O) is directly proportional to the concentration of protons in the sample. In 3 per cent agar gels there is observed an apparent decrease in the area under the curve of the water signal compared to 100 per cent water standard at the same setting, suggesting that about half of the protons of the water are not being registered. Most or all of the "missing" water signal, however, becomes apparent on increased amplification and appears in the "wings," the increased line width of agar gels remaining constant. The increased line width in agar is not due to saturation of the system of protons by the rf (radiofrequency) field, since the line width of water in 3 per cent agar was found to be independent of the rf intensity normally used.

The line width of agar gels was markedly influenced by temperature. On heating a sample in a boiling water bath and then recording spectra as the agar is cooling, changes are observed, the water signal in hot agar sols resembling free water and becoming broader as it gels. At temperatures of 63° to 48°C, 3 per cent agar shows a line width of 2 cps; upon further cooling to room temperature it achieves its final broadened state. This finding demonstrates that a paramagnetic impurity in the agar is not responsible for line broadening since such a factor should be operative independent of temperature. T_1 in 3 per cent agar gels prepared in distilled water is of the same order as that of pure water. Although no direct determinations of T_2 were made, the "ringing" observed on the oscilloscope with H_2O, is markedly reduced in the agar samples, as shown in Figure 1, indicating that T_2 is decreased relative to pure water.

The broadening observed in agar gels is highly specific. Gelatin gels prepared in water (5 to 25%) do not show significant broadening of line width. Methocel solutions (2%), ranging in absolute viscosity from 15 to about 7,500 centipoises at 20°, as well as their gels (prepared by heating) did not exhibit the broadening. Similarly, 2 per cent solutions of sodium carboxymethylcellulose (CMC), ranging in absolute viscosity from 35 to about 40,000 centipoises (at 25°) did not exhibit line widths greater than 4 cps, even though high viscosity CMC sets up to form a thixotropic gel. Viscous solutions of PVP (40–50%) and pectin (5%) likewise have only slight effects on line width of the water signal. Balazs *et al.*[8] who studied a great variety of other gel systems, including collagen, hyaluronic acid, myosin, and gelatin, have reported no broadening of the water signal. Accordingly, the line broadening observed with agar cannot be explained in terms of any simple gelation or viscosity effect.

Other differences between agar gels and the other gel systems mentioned were evaluated. For example, it is possible to consider that there are relatively large

"lakes" in gels like gelatin whereas in agar gels the matrix might be so arranged that the water was enclosed within small heterogeneous compartments, which might theoretically give rise to the broadening observed with agar. It is also possible to consider that as agar sets to form a gel, diamagnetic anisotropy is introduced, whereas with the other gels this does not occur. These possible explanations for line broadening were examined by studying the behavior of the CH_3 protons of tetramethylammonium chloride added to agar; these protons should be subject to the same influences as the protons of water in the gel. Agar gels (3%) prepared in 1 *M* tetramethylammonium chloride show the characteristic broaden-

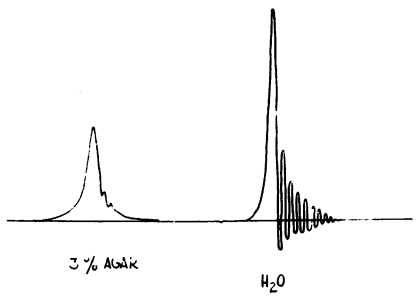

FIG. 1.—The oscilloscope tracings of high resolution proton magnetic resonance spectra of 3 per cent agar gel and of pure water at the same settings, illustrating relaxation wiggles (ringing). A decrease in ringing is associated with a decrease in T_2, the spin-spin relaxation time, which is a measure of the rate at which the nuclear moments transfer energy to one another, either directly with each other, or with their environment.

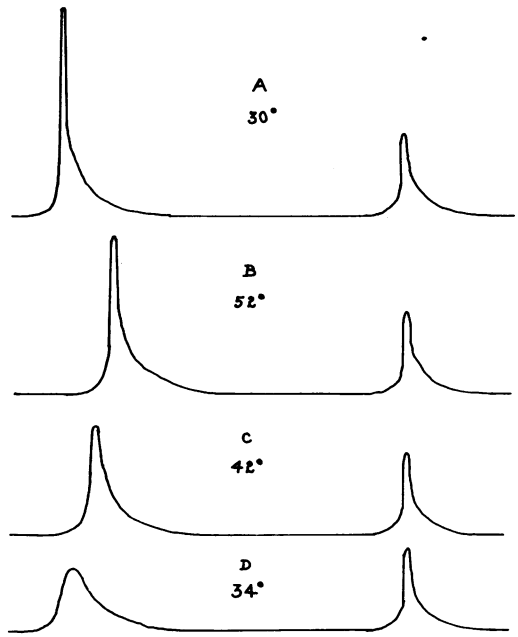

FIG. 2.—The recorded NMR spectra at a constant setting of 1*M* tetramethylammonium chloride in 20 per cent H_2O–80 per cent D_2O at 30° (*A*), and 3 per cent agar gel made up in the same aqueous system at 52°(*B*), 42°(*C*) and 34°(*D*); the water peak is at the left, 89.7 cps. "downfield" from the methyl peak at room temperature (30°) in both water and 3 per cent agar. Note that in 3 per cent agar, the methyl peak remains constant in line width and peak amplitude, while in agar the water peak increases in line-width and decreases in peak amplitude as temperature decreases and gelation occurs.

ing of the water peak; however, the methyl peak (89.7 cycles upfield at room temperature) is not significantly broadened. Moreover, on heating such agar gels and recording spectra while cooling, it is observed that the methyl peak shows no significant change as the temperature of the gel is varied, while the water peak is sharpened at high temperatures and broadens as temperature is lowered. Figure 2 shows a typical experiment of this type; to obtain tracings which show both the water peak and the methyl peak at the same level of amplification during the course of the run, the H_2O signal in the experiments shown are those obtained using

1 *M* tetramethylammonium chloride in 20% H_2O–80% D_2O, so as to decrease the H_2O peak.[10] (Similar results are obtained if 100 per cent water is used, except that in this case it is necessary to increase amplification after the water peak has been recorded to see the characteristics of the CH_3 peak.) Since heterogeneous compartmentalization of water, diamagnetic anisotropy of the system, or the presence of paramagnetic impurities should influence the protons of water and methyl to an equivalent extent, the line broadening of the water peak in agar is not explicable in terms of these factors. There remains the possibility that the line broadening is due to decreased mobility of water protons in agar gels relative to pure water, implying the existence of water structures intermediate in rigidity between free water and ice.

It must be emphasized that the water in agar gels does not appear to be a two phase system consisting of a mixture of "free" water and water in some other state. On high amplification there is no evidence for discontinuity of the water signal in agar gels; moreover, if a capillary containing "free" water is introduced into a 3 per cent agar gel, this free water sample registers as a superimposed peak to the "low field" side of the agar-water signal (together with its ringing), suggesting that if a large fraction of free water had actually been present in the gel, this might have been registered as a peak superimposed on a wider signal (Fig. 3).

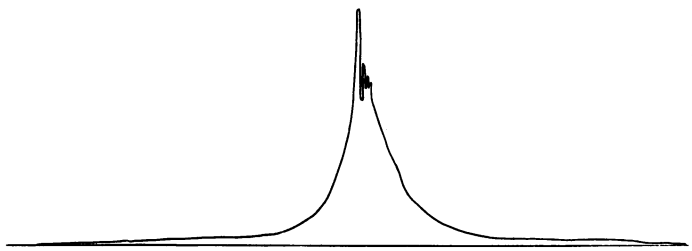

FIG. 3.—A high amplification recorded spectrum of a 3 per cent agar gel containing a capillary filled with pure water. The water in the capillary registers as the sharp peak, with ringing, to the left (downfield) of the peak of the bulk water present in 3 per cent agar.

The present results, which indicate that the water in an agar gel at room temperature is in a state different from that of ordinary aqueous solutions, can be interpreted along lines discussed by Bernal.[1] Thus, it may be considered that at the surface of the polysaccharide chains in 3 per cent agar, water molecules are hydrogen-bonded to hydroxyl groups (on a 1:1 or 2:1 basis) resulting in a rigid "ice-like" arrangement of a small number of the water molecules. The bulk of the water between polysaccharide chains, which registers in NMR, would be subject to so-called long range forces and be in a state intermediate between an ice-like arrangement and "free" to the extent that it would possess a certain degree of rigidity reducing its mobility, while in large part retaining the solvent properties of free water. Such an explanation could account for the fact that the water in agar gels, while appearing to be "free" to act as a solvent for tetramethylammonium chloride, nevertheless appears to be "ordered" to a higher degree than "free" water. The specificity of agar among a variety of polysaccharides and gels indicates the importance of some structural feature of the gel matrix which appears to be neces-

sary for the "ordering" of water molecules. The specific mechanism involved, as yet unknown, merits serious investigation.

Summary.—The proton signal of the water in agar gels, studied in high resolution NMR, differs from that of pure water in that the line width is significantly broadened and the amplitude is decreased; T_1 is not influenced but T_2 is decreased. This effect in agar is not shown by a variety of other gels and viscous solutions studied. The signal of the methyl protons from tetramethylammonium chloride is essentially equivalent when studied in agar gels or in pure water in marked contrast to the proton signal from water. These findings demonstrate that a possible heterogeneity of the internal field, resulting from compartmentalization of water, diamagnetic anisotropy, or the presence of paramagnetic impurities, are not responsible for the NMR changes observed in the water of agar gels. The NMR data can best be explained at present on the basis that water in an agar gel is in a modified state with properties of structural rigidity and mobility intermediate between "free" water and ice.

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[10] There is a chemical shift as temperature is varied in the 3 per cent agar, the water signal shifting downfield as temperature decreases (methyl peak as standard); similar results are obtained, however, with water-tetramethylammonium chloride. The similar chemical shift observed with both 3 per cent agar and water relative to the methyl signal does not necessarily mean that the degree of hydrogen bonding of water in the two cases is necessarily the same.

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*STUDIES ON BEEF SPLEEN CATHEPSIN A**

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Previous studies on the action of proteolytic enzymes in animal tissues (spleen, kidney, liver) on synthetic substrates for well-defined proteinases (pepsin, trypsin, chymotrypsin) have led to the identification of three cathepsins, designated A, B, and C, respectively.[1] Cathepsin A (termed cathepsin I in earlier papers[2–4]) was characterized by its optimal action on carbobenzoxy-L-glutamyl-L-tyrosine