A STUDY OF THE INFLUENCE OF WATER ON THE DENATURATION OF DEOXYRIBOSE NUCLEIC ACID

by

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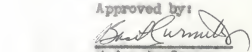

Major Professor

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INTRODUCTION

In 1953, Watson and Crick (1) proposed a model for DNA as consisting of a double-stranded helix comprised of two right-handed single helical chains of nucleotides running in opposite directions. This model is generally accepted today for the B form or high-humidity form of DNA. The double helix has a pitch of 34A with 10 residues per turn in each chain. Inside the helix, the bases are stacked 3.4A apart, almost perpendicular to the fiber axis, and are specifically paired by hydrogen bonds. Adenine on one chain with thymine on the other, and guanine on one chain with cytosine on the other. The sequence of bases in one chain uniquely determines the sequence of the other.

Simply heating the double-stranded DNA breaks the base pair hydrogen bonds and partially or completely separates it into two strands of "denatured" DNA. Denaturation refers to the disorganization of the double-helical or secondary structure of DNA. Heating a solution of native DNA produces little change in physical properties such as viscosity or ultraviolet absorption until the denaturation temperature is reached. At this temperature a decrease in viscosity and a 40% increase in ultraviolet absorption occurs. The absorption increase takes place over a narrow temperature range indicative of a cooperative transition. This transition is similar to melting, except that the absorption versus temperature change is irreversible.

The extent of the role of the solvent on helix stability of DNA has not at present been well established. Numerous studies indicate that DNA is more stable in aqueous solutions than is reasonable to expect in view of high concentrations of the competitive hydrogen-bond acceptor and

donor water. It is believed that hydrogen-bonded base pairs in the interior of the double helix provide selectivity but only a fraction of the stability of DNA. If in aqueous solutions hydrogen bonds supply only a fraction of the stabilization energy of the double-helical DNA structure, other explanations must be advanced for helix stabilization. Possibilities are electrostatic forces, van der Waals forces, and hydrophobic forces.

The purpose of this research is to study solvent effects on DNA mainly through the use of nuclear magnetic resonance techniques.

Theory of Nuclear Magnetic Resonance

An outline of Bloch's macroscopic theory will be used to present the theory for nuclear magnetic resonance. (2)

We investigate the behavior of a large number of nuclei contained in a macroscopic sample of matter and acted upon by two external magnetic fields; a strong constant field and at right angles to it, a comparatively weak radio-frequency field. Let the z -direction be that of the constant magnetic field with strength H_0 and the x-direction that of the radio-frequency field with angular frequency ω and amplitude $2H_1$, therefore the total external field vector $\underline{H}$ has the components: $H_x = 2H_1 \cos \omega t$, $H_y = 0$ and $H_z = H_0$.

If we consider a nucleus of magnetic moment μ in a magnetic field H, the nuclear magnet experiences a torque $(\underline{\mu} \times \underline{H})$, which must be equated to the rate of change of angular momentum. Since the angular momentum of the nucleus is $\frac{\mu}{\gamma}$, we have $\frac{1}{\gamma} \frac{d\underline{\mu}}{dt} = \underline{\mu} \times \underline{H}$. (1)

If now we consider an assembly of weakly interacting nuclear spins,

then the magnetization vector M is the vector sum of the nuclear magnetic moments in a unit volume. Summing (1) over unit volume, we get

$$\frac{d\mathbf{M}}{dt} = \gamma \mathbf{M} \times \mathbf{H} , \qquad (2)$$

which gives the change in orientation of the magnetization vector due to the presence of external fields alone.

These considerations are only qualitative however since we have neglected effects due to atomic electrons and neighboring nuclei.

The importance of atomic moments depends upon the substance under consideration. There are many substances, water for example, where the electron spins are paired off and their effect can therefore be justifiably neglected. Many substances have permanent atomic moments and in such cases these moments cannot be neglected; however, we shall not consider substances with permanent atomic moments.

In order to understand the effects of neighboring nuclei, we must consider nuclear relaxation. There are two types of relaxation. The first, has to do with the establishment of thermal equilibrium between an assemblage of nuclear magnets with different quantum numbers. This is "longitudinal" relaxation, since it results in establishment of an equilibrium value of the nuclear magnetization along the magnetic field axis. An assemblage of nuclei in a very weak magnetic field, such as the earth's magnetic field, will have essentially no net magnetization of the nuclei along the field axis, since only a few more of the nuclei possess the spin quantum number $(+\frac{1}{2})$ as compared with those with the value of $(-\frac{1}{2})$. When this assemblage is placed in a magnetic field and relaxation takes place, there is an increase in the sample magnetization along the field axis as more of the nuclei drop into the lower energy

state with magnetic quantum number of $(+\frac{1}{2})$. The characteristic longitudinal relaxation time is designated as T_1.

The property of magnetic nuclei which corresponds to precession provides a means whereby energy may be transferred back and forth between the nuclei and their surroundings and promotes longitudinal relaxation. An important mechanism for relaxation of a group of nuclei at a non-equilibrium spin temperature utilizes atomic and molecular thermal motions as follows: If a magnetic nucleus is surrounded by others of its type contained in atoms undergoing violent thermal motions, the thermal motions of the nuclei produce random oscillatory magnetic fields which can have frequency components with frequencies equal to the precession frequencies of the relaxing nuclei and can act as a rotating magnetic field vector so as to permit the magnetic orientation energy to be converted to thermal energy. The rate of relaxation by this mechanism depends on the temperature, the concentration of magnetic nuclei, and the viscosity of the medium. Thermal motions of substances with unpaired electrons are particularly effective in inducing thermal relaxation, and such paramagnetic substances provide an effective means by which the "longitudinal" relaxation time can be reduced.

Since "longitudinal" relaxation results in the establishment of thermal equilibrium between an assemblage of nuclear magnets with different quantum numbers, there must be an exchange of energy between the nuclei and their surroundings (or so-called lattice). "Longitudinal" relaxation is sometimes called "spin-lattice" relaxation.

The other type of relaxation may be illustrated as follows: If we consider a group of nuclei precessing in phase about the axis of a

common magnetic field and if the nuclei were bunched close enough together as to be considered centered at the same point, their magnetic vectors would be precessing together like a tied-up bundle of sticks. If we take the magnetic field axis to be the Z-axis, the nuclei precessing in phase at resonance produce a resultant rotating magnetic vector which has a component in the x-y plane. If by some process the nuclei tend to lose their phase coherence, their resultant will move toward the Z-axis and the macroscopic components of magnetization in the x-y plane will go to zero. This type of relaxation is commonly referred to as "transverse" relaxation, and its rate is expressed in terms of the characteristic time T_2.

There are several factors which contribute to transverse relaxation. The homogeneity of the applied magnetic field will be extremely important as an external factor. If the assemblage of nuclei under consideration is in a nonhomogeneous field, the nuclei will not have identical precession frequencies, and if they start off in phase, they will soon get out of phase because of their different precession rates. In liquids, with low viscosity, the inhomogeneity of the applied magnetic field will generally be the most important factor determining T_2.

For solids or viscous liquids, spin-spin interactions are important contributors to T_2. One type of spin-spin interaction occurs when two identical nuclei exchange spin quantum numbers, one nucleus providing a rotating field vector for the other. The exchange of spin quantum numbers limits the time of maintenance of phase coherance for a group of identical nuclei precessing in phase and therefore decreases T_2. Another effect is due to each nuclear magnet being in a different magnetic field. Each nuclear magnet finds itself not only in the applied magnetic

field H_o, but also in a small local magnetic field H_{local} produced by the neighboring nuclear magnets. Since the magnetic field, as seen by each respective nucleus differs from nucleus to nucleus, there will be a distribution of the frequencies about the Larmor precession frequency, covering a range Δw, which is equal to γH_{local}. In liquids with low viscosity, the molecules tumble rapidly relative to the precession frequencies of their nuclei, the fluctuations in the local magnetic fields are effectively averaged to zero, therefore this factor does not contribute to T_2 in liquids with low viscosity.

In the first part of the development of the theory, we considered means by which the magnetization vector could be reoriented because of external fields. Now we must also consider the change in the magnetization vector due to the interaction between neighboring nuclei. It will be assumed that the change in the longitudinal component M_z due to interaction between neighboring nuclei is

$$\frac{dM_z}{dt} = -\frac{(M_z - M_o)}{T_1} \quad , \tag{3}$$

and the change in the transverse components of the magnetization M due to interaction between neighboring nuclei is given by

$$\frac{dMx}{dt} = -\frac{M_x}{T_2} \qquad \frac{dMy}{dt} = -\frac{My}{T_2} \quad . \tag{4}$$

The total rate of change of M is obtained by adding the terms due to the action of the external field to the terms due to action of neighboring atoms, summing we get the Bloch equations

$$\frac{dM_x}{dt} = \gamma(M_y H_z - M_z H_y) - \frac{M_x}{T_2} \quad ,$$

$$\frac{dM_y}{dt} = \gamma(M_z H_x - M_x H_z) - \frac{M_y}{T_2} \quad , \text{ and} \qquad (5)$$

$$\frac{dM_z}{dt} = \gamma(M_x H_y - M_y H_x) - \frac{(M_z - M_0)}{T_1} \quad .$$

If we replace the radio-frequency field, $H_x = 2H_1 \cos \omega t$, $H_y = 0$, by the component which causes transitions between the energy levels of the nuclei; then, $H_x = H_1 \cos \omega t$, $H_y = \mp H_1 \sin \omega t$, $H_z = H_0$.

Consider the projection of $\underline{M}$ on the x-y plane, M_{xy}. Movement of M_{xy} so as to produce a change in M_y will cause a current to be induced in a receiver coil mounted along the y-axis. If we consider M_{xy} to be made up of two magnetic components u and v, one in phase with H_1 and one out of phase with H_1, respectively, so that $M_{xy} = u + iv$. The components u and v can be defined by the equations:

$$u = M_x \cos \omega t - M_y \sin \omega t,$$
$$v = -(M_x \sin \omega t + M_y \cos \omega t). \qquad (6)$$

These equations in combination with the Bloch equations give:

$$\frac{du}{dt} = -(\omega_0 - \omega)v - \frac{u}{T_2} \quad , \qquad (7)$$

$$\frac{dv}{dt} = (\omega_0 - \omega)u - \frac{v}{T_2} - \gamma H_1 M_z \quad , \text{ and} \qquad (8)$$

$$\frac{dM}{dt} = \frac{(M_0 - M_z)}{T_1} + \gamma H_1 v \quad , \text{ where,} \qquad (9)$$

$$\omega_0 = \gamma H_0 \quad .$$

Equation (9) shows that the energy absorbed by the nuclei through changes in their magnetic quantum numbers with respect to H_o is a function of v and not of u. Therefore one must measure v if one desires a measure of the energy absorbed by the nuclei as a function of H_o at constant H_1.

If one assumes a steady-state condition, with H_o being held constant and a steady signal being picked up in the receiver coil, such as occurs when the magnetic field sweep is stopped on the side of or peak of a reference signal, then M_{xy} has a constant length and rotates about the z-axis at an angular frequency ω. The steady-state condition requires that,

$$\frac{du}{dt} = \frac{dv}{dt} = \frac{dM_z}{dt} = 0. \tag{10}$$

Under these conditions

$$u = T_2 (\omega - \omega_o) v,$$

$$v = \frac{\gamma T_2 H_1 M}{1 + T_2^2(\omega - \omega_o)^2}, \text{ and} \tag{11}$$

$$M = M_o \frac{1 + T_2^2 (\omega_o - \omega)^2}{1 + T_2^2 (\omega_o - \omega)^2 + (\gamma H_1)^2 T_1 T_2}$$

Substituting the equation for M_z into that for v gives

$$v = \frac{\gamma H_1 M_o T_2}{1 + T_2^2(\omega_o - \omega)^2 + (\gamma H_1)^2 T_1 T_2}, \text{ and} \tag{12}$$

$$u = \frac{\gamma H_1 (\omega_o - \omega) T_2^2 M_o}{1 + T_2^2(\omega_o - \omega)^2 + (\gamma H_1)^2 T_1 T_2}. \tag{13}$$

The peak height of the v-mode signal is,

$$v_{max} = \frac{\gamma H_1 T_2 M_0}{1 + (\gamma H_1)^2 T_1 T_2} \quad , \qquad (14)$$

which occurs when $(\omega_0 - \omega) = 0$.

The quantity v is a maximum when $\gamma^2 H_1^2 T_1 T_2 = 1$. At this power level, the signal is twice as broad as it would be at very low power levels and with further increase of H_1, it broadens still further and decreases in amplitude. This phenomenon is called "saturation". It may be thought of as arising when the nuclei are depolarized by the action of the radio-frequency field more rapidly than they can be repolarized through the longitudinal relaxation process.

The integral of the v-mode with respect to the magnetic field strength H is,

$$\int v \, dH = \frac{K M_0 H_1}{\left[1 + \gamma^2 H_1^2 T_1 T_2\right]^{\frac{1}{2}}} \quad , \qquad (15)$$

where K = constant

M_0 = Nuclear Magnetization,

$M_0 = \chi_0 H_0$,

$\chi_0 = \frac{I + 1}{3I} \frac{N\mu^2}{KT}$

I = nuclear spin number,

N = number of nuclei in the volume of the receiver coil,

μ = nuclear magnetic moment,

K = Boltzmann's constant,

and T = absolute temperature.

If we assume a linear sweep of magnetic field at a rate r and integrate the v-mode χ_s. time, we obtain the following expression for the area under the absorption mode:

$$\text{Area} = \frac{C}{T}\,\frac{H_1}{r}\,\frac{N}{\left[1+\gamma^2 H_1^2 T_1 T_2\right]^{\frac{1}{2}}} \quad , \qquad (16)$$

where c includes the various nuclear and instrumental constants, T is the absolute temperature, H_1 is the radio-frequency driving field, and the expression in brackets shows the dependence upon relaxation times. Only when the relaxation times are very short, or the radio-frequency power level is very small, so that $(\gamma H_1)^2 T_1 T_2 << 1$, does the area become independent of the relaxation times for "slow passage" conditions. Experimentally, in order to meet "slow passage" conditions, the time spent in traversing the resonance must be comparable to or greater than the inverse of $\Delta\omega$, where $\Delta\omega$ is the half-amplitude line width in units of angular frequency. The time rate of change of the scanning field must therefore decrease in proportion to the square of the line width observed.

To determine the effects of varying sweep rates, power levels, and relaxation times, Williams (3) solved the v-mode integral using an electronic digital computer for a wide range of values of $\frac{\gamma H_1^2}{\frac{dH}{dt}}$ and slow passage saturation parameter, $(\gamma H_1)^2 T_1 T_2$. He assumed that the experimentally observed transient oscillatory decay following rapid passage through resonance is due to an oscillatory term which makes a negligible contribution to the integral and is superimposed upon a non-oscillatory term which is equal to the slow passage result. This assumption is borne

out by the treatment of Jacobsohn and Wangsness (4) and is proven by experiment.

If we look at certain limiting conditions in determining the area of the absorption mode, for $(\gamma H_1)^2 T_1 T_2 \ll 1$, the term $\left[1 + (\gamma H_1)^2 T_1 T_2\right]^{-\frac{1}{2}}$ can be expanded in the form $1 - \frac{1}{2}(\gamma H_1)^2 T_1 T_2 + \ldots\ldots$, which suggests that the excess population of polarized nuclei, N, producing resonance is effectively reduced by an average percentage $\frac{1}{2}(\gamma H_1)^2 T_1 T_2$ during passage through resonance. If we regard the quantity $(\gamma H_1)^2 T_2$ as the transition probability per unit time for the nuclei and the quantity T_1 as the time during which transitions are permitted to occur in order to meet the requirements for slow passage. The product of these terms is a measure of the reduction in the population during the time of observation.

For "rapid passage" through resonance, T_1 can be modified by τ, the actual time during which transitions are permitted to take place, or the time spent in traversing the resonance, $\tau = \frac{\Delta H}{\frac{dH}{dt}}$, replacing the line width, ΔH, by the equivalent expression $\Delta H = \frac{1}{\gamma T_2}$, we obtain upon substitution of τ for T_1,

$$\int v dt = \frac{K H_1 N}{T \frac{dH}{dt}\left[1 + \frac{\gamma H_1^2}{\frac{dH}{dt}}\right]^{\frac{1}{2}}} \quad (17)$$

This can be rewritten as,

$$\int v dt = \frac{K H_1 N}{T \frac{dH}{dt}}\left[1 - \frac{1}{2}\frac{\gamma H_1^2}{\frac{dH}{dt}} + \ldots\ldots\right], \text{ for } \frac{\gamma H_1^2}{\frac{dH}{dt}} \ll 1. \quad (18)$$

We see from the above equation that for $\frac{\gamma H_1^2}{\frac{dH}{dt}} << 1$, the areas can be made independent of relaxation times. Therefore it is not always necessary to operate under "slow passage" conditions in order to have absorption areas independent of relaxation times of the various nuclei.

Field-dependent differences between magnetic resonance line positions arise because the lines of force of the applied magnetic field tend to be turned away from the nuclei by a diamagnetic shielding effect of the surrounding electrons. These field-dependent differences are called "chemical shifts". The degree of diamagnetic shielding is directly proportional to the applied field. The possibility of applying proton resonance measurements for studying hydrogen bonding in molecules became apparent following the observation by Arnold and Packard (5) that the position of the proton signal of the OH groups in the alcohol spectrum were temperature and concentration dependent. Increase of temperature or increasing dilution caused the signal to shift to high field. On forming a hydrogen bond, the hydrogen of the OH group may be expected to experience a different electron shielding from that in the free or non-associated state.

Previous Studies of DNA-Water Mixtures Using NMR Techniques

Although the area under the absorption curve for water is proportional to the number of water protons in the receiver coil, the protons in ice absorb over a very wide range of the magnetic field because of variations in H_{local} and, consequently, in a water-ice mixture do not contribute measurably to the area of the narrow water proton signal. It is possible to use this technique for the detection of the postulated ice-like domains of water in the presence of macromolecules.

Jacobson, Anderson, and Arnold (6) measured the areas under the absorption curves for DNA-water mixtures as compared to the areas under the absorption curves for water alone. They measured a decrease in area for 1.6% DNA as compared to water. They further suggested that a considerable fraction of the water in such a solution is in a bound, lattice-ordered (ice-like) form.

Balazs, Bothner-By, and Gergely (7) did further work on DNA-water mixtures and found no area differences between DNA-water mixtures and water itself. They further suggested that the difference in areas obtained by Jacobson, Anderson, and Arnold (6) was due to an instrument artifice and not due to water taking on a bound, lattice-ordered form.

Both Jacobson, Anderson, and Arnold (6) and Balazs, Bothner-By, and Gergely (7) observed a broadening of the water signal in the presence of DNA. For 2% DNA, Balazs, Bothner-By, and Gergely obtained half-amplitude line widths twice as broad as for water alone.

Balazs, Bothner-By, and Gergely (7) also studied nuclear magnetic resonance of the water proton in the presence of other macromolecules. Broadening of the water signal was not observed for other macromolecules however. This difference in behavior led to the postulate that a large diamagnetic anisotropy produced by the stacked planar arrangement of purine and pyrimidine bases in the deoxyribose nucleic acid molecule is responsible for the observed effect. In order to test this hypothesis, an examination of the behavior of water protons in graphite suspensions was made. Such suspensions showed a broadening even more pronounced than that observed in DNA solutions.

The observed broadening of the water signal in the presence of DNA could be interpreted in terms of a small hydration shell. The protons

in this shell would have a longer correlation time than the free protons, and consequently, they would have a much shorter nuclear magnetic transverse relaxation time T_2. If the rate of molecular exchange between water in the water phase and that in the hydration shell occurs at high frequencies compared with the magnetic relaxation time of the free protons, then broadening of the line is expected, since the nuclear spins of the protons entering the hydration shell would exchange their energy with those of the bound protons. This mechanism would lead to a decrease in the transverse relaxation time, T_2, the measure of the time during which initially synchronous precessing spins get out of phase. According to this mechanism, no change in T_1, characteristic of the exchange of energy between the proton nuclear spins and the lattice would occur. This mechanism, then, would account for the observed broadening, for the constancy of the area under the peak, and for the constancy of T_1. However, the fact that no changes were observed in the water signal of other macromolecules in the same concentration forming similar gels suggests that if this mechanism is responsible for the changes observed with DNA, the hydration of DNA must depend on a special mechanism. (7)

Further studies of DNA-water mixtures using NMR techniques were done by Campbell, Mahler, and Moore. (8) A line splitting of 1.67 cycles per second was observed for native DNA. This effect vanished progressively with longer periods of sonication and also disappeared after formaldehyde denaturation. The results were interpreted as indicating the presence of an ice-like hydration shell several molecular layers thick.

Experimental Apparatus

The NMR spectrometer used was a Varian, Model A-60 Analytical spectrometer. The field intensity of the applied field H_o is 14,092 gauss while the rotating field frequency is set at 60 Mc. The resolution of the instrument is (1 part in 10^8) or .6 cycle/sec full line width at half amplitude with sample spinning.

The Varian A-60 Analytical NMR spectrometer uses the single-coil system with a number of variations. Two samples, rather than one are used; a control sample of water which is a permanent part of the probe, and an experimental sample which is inserted by the operator for observation. Both the control sample and the experimental sample are excited by a rotating magnetic field of 60 Mc, H_1, and by a 5 Kc modulation of the H_o field produced by modulating coils in the same region. The effects of the 5 Kc modulation of the H_o field is to introduce a second rotating field which, as seen by the nuclei, is equivalent to modulating the 60 Mc rotating field, producing sideband components 5 Kc above and below the 60 Mc fundamental frequency. In the model A-60, the fundamental H_o field intensity is adjusted to a value, which according to the Larmor equation, will cause the nuclei to precess at the upper sideboard frequency. The fundamental H_o field is never adjusted for precession of the nuclei at 60 Mc, hence the system is said to be "tuned" to the upper sideband.

In order to achieve resonance, a d-c sweep coil, surrounding the experimental sample only, is included in the probe. A sweep current is passed through this coil to change the H_o field slowly in the region of the experimental sample across the value required for resonance at 60 Mc plus 5 Kc. The current amplitude in the coil is accurately calibrated

in terms of precession frequency displacement from a known reference spectral line, Tetramethylsilane, and is accurately related to the pen-carriage position of a graphic recorder.

In the Model A-60, an air-turbine sample spinner is used to average the effects of any small field inhomogeneities which may exist, thereby improving the effective system resolution. Sample spinning is at the rate of approximately 25 r.p.s.

A Varian V-6057 Variable Temperature System was used with the A-60 spectrometer. The High Temperature system used provides control of sample temperature between +40° C and +200° C, with temperature regulation to ±2° C at the sample.

EXPERIMENTAL PROCEDURE, THEORY, AND CALCULATIONS

Half-amplitude line widths were measured for different water mixtures at 25° C with the results shown in Table I. The results agreed with those published by Balass, Bothner-By, and Gergely. (7) The graphite and DNA solutions had a broadening effect on the water resonance spectra while the gelatin gel and salt solutions had no effect. However, the half-amplitude line width for 1.6% DNA was less than obtained by Balass, Bothner-By, and Gergely.

The relative values of the product T_1T_2 were measured for water and 1.6% DNA at 25° C and for 1.6% DNA concentrations at higher temperatures. The progressive saturation method was used for these measurements. The reader is referred to Andrew (9) for a detailed description of this method. The amplitude of the absorption mode was measured for different values of the radio-frequency field under saturation conditions. In

Table I. Relative Line Widths of Different Water Mixtures

SAMPLE	RELATIVE LINE WIDTH (a)
5% gelatin gel	1.0
2% graphite in 5% gelatin gel	5.4
1.6% DNA	1.2
1% sodium chloride	1.0
10% sodium chloride	1.0

(a) The relative line width is the ratio of the line width of the solution to that of the solvent. The solvent was distilled water. The error in the measurements of the relative half-amplitude line widths was approximately $\pm$ .1 cycles/sec.

Plate I, saturation curves are shown for water and 1.6% DNA at 25° C. For any horizontal line drawn on these curves,

$$(\gamma^2 H_1^2 T_1 T_2)_{water} = (\gamma^2 H_1^2 T_1 T_2)_{1.6\%\ DNA} , \qquad (1)$$

therefore

$$\frac{(T_1T_2)_{1.6\%\ DNA}}{(T_1T_2)_{water}} = \frac{(H_{1_{water}})^2}{(H_{1_{1.6\%\ DNA}})^2} . \qquad (2)$$

By drawing a horizontal line at half-amplitude,

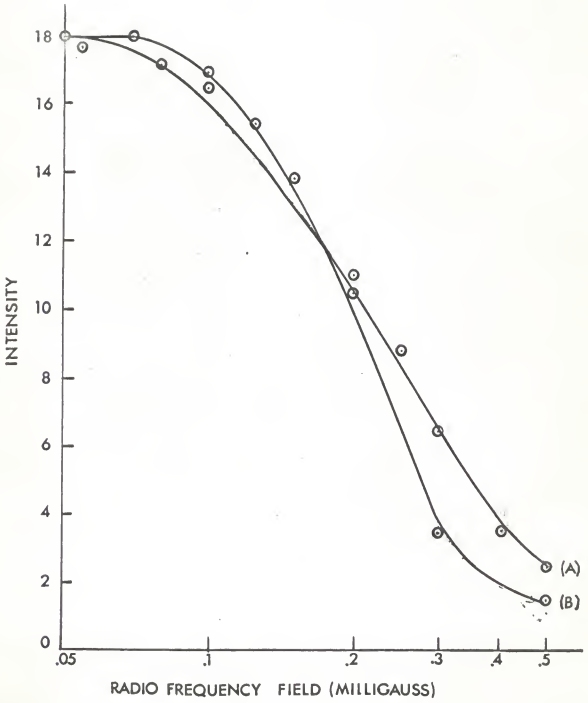
$$\frac{(T_1T_2)_{1.6\%\ DNA}}{(T_1T_2)_{water}} = .79 \text{ at room temperature.}$$

In Plate II, saturation curves are shown for 1.6% DNA at 25° C, 59° C, and 86° C respectively. By drawing a horizontal line at half-amplitude, the

EXPLANATION OF PLATE I

Intensity vs. radio-frequency field for water and 1.6% DNA at room temperature. (A), 1.6% DNA; (B), Water.

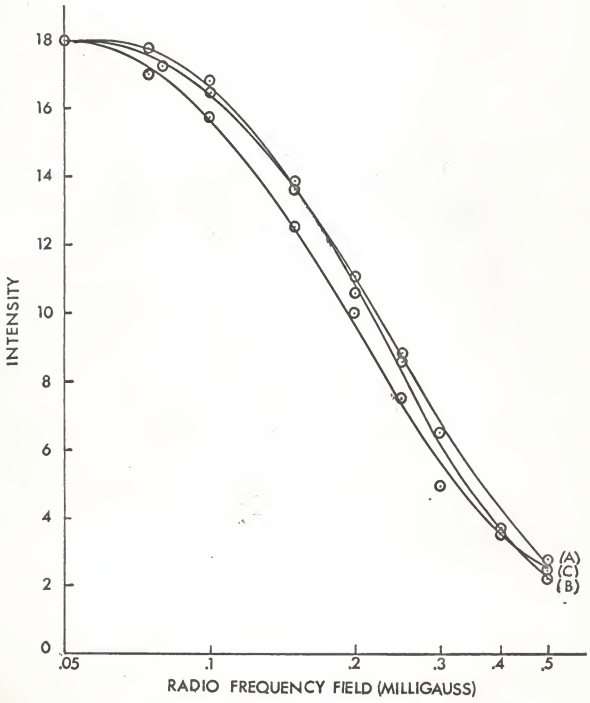
PLATE I


18
16
14
12
10
8
6
4
2
0
INTENSITY
.05
.1
.2
.3
.4
.5
RADIO FREQUENCY FIELD (MILLIGAUSS)
(A)
(B)

EXPLANATION OF PLATE II

Intensity vs. radio-frequency field for 1.6% DNA at three different temperatures. (A), 1.6% DNA, 86° C; (B), 1.6% DNA, 59° C; (C), 1.6% DNA, 25° C.

PLATE II


INTENSITY
0
2
4
6
8
10
12
14
16
18
.05
.1
.2
.3
.4
.5
RADIO FREQUENCY FIELD (MILLIGAUSS)
(A)
(C)
(B)

product T_1T_2 was obtained. The results are shown in Table II.

Table II. Relative T_1T_2 Values for 1.6% DNA at 59° C and 86° C as compared to T_1T_2 for 1.6% DNA at room temperature.

TEMPERATURE	RELATIVE T_1T_2
59° C	1.08
86° C	1.28

Further evidence that the product T_1T_2 does not vary appreciably with increase in temperature for 1.6% DNA was obtained by measuring the areas under the absorption mode at different temperature for both "rapid passage" and "slow passage" conditions. Under "rapid passage" conditions, i. e., for $\frac{\gamma H_1^2}{\frac{dH}{dt}} \ll 1$, the areas are independent of relaxation times. However, for "slow passage" conditions, the areas are governed by the factor $(\gamma H_1)^2 T_1T_2$, providing the condition $(\gamma H_1)^2 T_1T_2 \ll 1$ does not hold. If the product T_1T_2 varies appreciably with increase in temperature, the slopes of the area versus temperature curves should vary for "slow passage" and "rapid passage" conditions. From Plate III, only a small change in slope was obtained from the area versus $\frac{1}{T}$ curve corresponding to "slow passage" and "rapid passage" conditions.

In order to test the hypothesis that a considerable fraction of the water in a DNA solution could exist in a bound, lattice-ordered (ice-like) form, the total area under the absorption mode was measured for six different DNA concentrations ranging from 1% to 10%. These areas were

EXPLANATION OF PLATE III

Area under the absorption mode for 1.6% DNA as a function of $\frac{1}{T}$ for both "rapid passage" and "slow passage" conditions. (A), 1.6% DNA, 500 sec. sweep time; (B), 1.6% DNA, 25 sec. sweep time.

PLATE III


(A)
(B)
76
72
68
64
60
AREA (ARBITRARY UNITS)
27
28
29
30
31
32
33
$\frac{1}{T}$ ($^{\circ}K^{-1}$) X 10^{-4}

compared with the total area under the absorption mode for water. Total areas were measured over a magnetic field width of 7.5 milligauss. All measurements were made under "slow passage" conditions. The radio-frequency field H_1, was reduced to such a value that the "saturation" factor $\gamma^2 H_1^2 T_1 T_2$ was much less than one. A planimeter was used to measure all areas. The uncertainty in the measurement of areas was approximately ±5%.

The DNA used in all nuclear magnetic resonance measurements was sodium salt DNA prepared from calf thymus. The DNA was purchased from Sigma Chemical Company. All DNA concentrations were prepared in a .15 M sodium chloride plus 1% sodium citrate solution. The DNA was dissolved in solution at 05° C. Samples were stirred for 24-48 hours at 05° C by the use of a wrist-action shaker. Samples were then freed of dissolved oxygen by evacuation with an aspirator. It was observed that air bubbles in the DNA solutions caused broadening and line-splitting of the resonance spectra at high temperatures. All DNA concentrations were measured by weight percentage to that of the solvent.

There was no measured decrease in area of the absorption mode of DNA-Water mixtures as compared to water. Because no area decrease was obtained, the theory that a considerable fraction of the water in such a solution is in a bound, lattice-ordered (ice-like) form seems to be disproved.

In Plate IV, Figure 1, area under the absorption mode of DNA-Water mixtures compared to the area of water is plotted versus the concentration of DNA. The area under the absorption mode remains relatively constant for concentrations of DNA ranging from 1% to 10% over a magnetic field

EXPLANATION OF PLATE IV

Fig. 1. Relative area of absorption mode for DNA-Water mixtures to area for water at different DNA concentrations.

Fig. 2. Half-widths of absorption mode of DNA-Water mixtures at different DNA concentrations.

PLATE IV

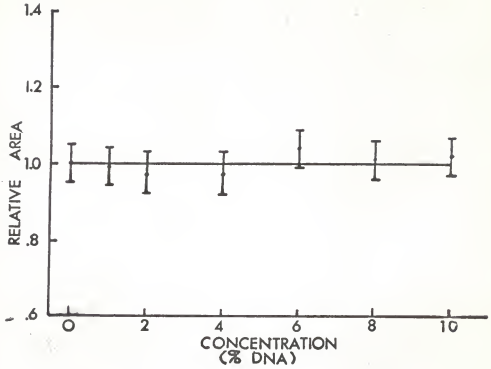


Fig. 1

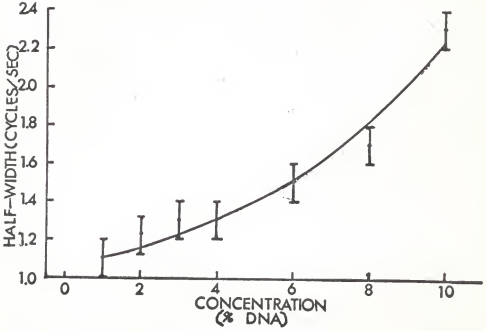


Fig. 2

width of 7.5 milligauss. The area under the absorption curve would be expected to decrease with increasing DNA concentrations for two reasons: (a) DNA displaces some water and, (b) any water bound to DNA in an ice-like form would not contribute to the observed proton resonance peak. The lack of concentration effect on the areas of different DNA concentrations could possibly be attributed to protons of the DNA molecule contributing to the area under the absorption curve over a range of 7.5 milligauss. A decrease in area under the absorption curve with increase in concentration of DNA was observed under "rapid passage" conditions. However, these areas were measured over a much narrower magnetic field width. Areas under the absorption curves for low concentrations of DNA correspond very closely to the area for water alone under "rapid passage" conditions.

Half-widths were measured for different concentrations of DNA in water. The half-widths were measured by taking the width of the absorption curve at one-half amplitude. In Plate IV, Figure 2, half widths for various DNA-water concentrations are plotted versus the concentration of DNA. Half-widths increased almost linearly with increase in concentration for the various DNA-water mixtures.

The "chemical shift" was measured for different DNA-water mixtures ranging from 1% to 10% DNA. In all measurements of the "chemical shift", distilled, degassed water was used as an external reference. The water was placed in a thin cylindrical capillary tube inside the sample to be run. The .15 M NaCl concentration in the DNA-water mixtures caused the resonance signal to be displaced .4 $\frac{\text{cycle}}{\text{sec}}$ upfield. Studies of proton magnetic resonance in concentrated aqueous electrolytes have been discussed

by Schoolery and Alder (10) and Hindman. (11) In both cases, NaCl shifted the resonance peak of water to slightly higher magnetic field positions.

In Plate V, Figure 1, the "chemical shift" is plotted in terms of the frequency separation $\Delta\nu$, between the reference sample water, and the signal due to water in DNA-water mixtures versus the concentration of DNA. The "chemical shift" was obtained by measuring the peak to peak distance between the resonance signals corresponding to water in DNA-water mixtures and that due to water alone. Due to the closeness of the resonance peaks, the error due to base line drift was reduced. The shift is almost identical to water alone and linear with respect to concentration. Shifts are to low magnetic field positions with respect to water.

Shearing forces were applied to different DNA-water concentrations by the method of sonication. The usefulness of this technique depends in part on the assumption that shearing forces produce transverse cuts not accompanied by secondary alterations in molecular structure. (12) If this assumption is true, sonication should cause a lowering of molecular weight of the DNA molecule without causing a change in the secondary structure of the DNA molecule. A Branson Instrument, Model LS-73, probe sonifier was used for sonication purposes. Each sample was exposed for approximately thirty seconds to sonication. The effect of sonication on the half-width for various DNA-water concentrations can be seen in Table III. Sonication narrowed the half-amplitude line widths of the absorption spectra corresponding to DNA-water mixtures.

The "chemical shift" was also measured for various concentrations of DNA which were sonicated. In Table IV, the "chemical shift" is shown for various DNA-water concentrations of both sonicated and unsonicated

EXPLANATION OF PLATE V

Fig. 1. "Chemical shifts" of DNA-Water mixtures at different DNA concentrations in a .15 M NaCl, 1% sodium citrate solution.

Fig. 2. "Chemical shift" corrections due to differences in volume diamagnetic susceptibility between DNA-Water mixtures and water for different DNA concentrations.

PLATE V

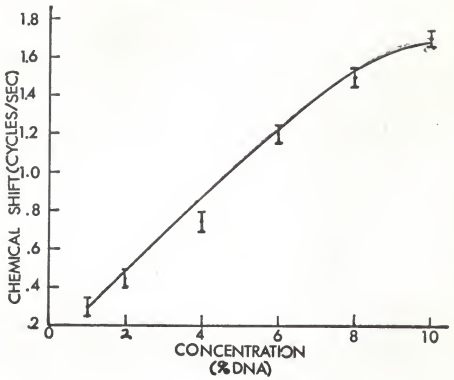


Fig. 1

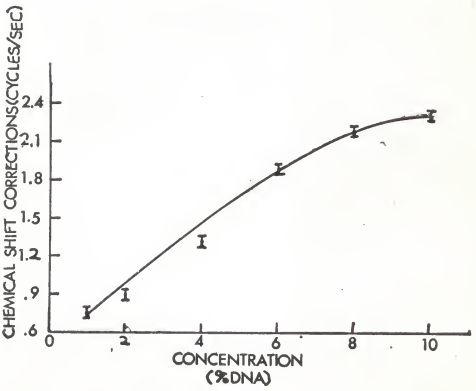


Fig. 2

samples. Distilled water was used as an external reference. Sonication decreased the peak-to-peak distance between resonance signals corresponding to water in DNA mixtures and that due to water in the external reference tube. Sonication produced the same effect as dilution in all cases.

Table III. Line Widths of Sonicated and Unsonicated DNA-Water Mixtures.

CONCENTRATION OF DNA	LINE WIDTH (SONICATED) $\left(\frac{\text{cycles}}{\text{sec}}\right)$	LINE WIDTH (UNSONICATED) $\left(\frac{\text{cycles}}{\text{sec}}\right)$
2%	1.1	1.2
6%	1.4	1.5
10%	2.0	2.3

Table IV. "Chemical Shifts" of Sonicated and Unsonicated DNA-Water Mixtures.

CONCENTRATION OF DNA	CHEMICAL SHIFT (SONICATED)(a) $\left(\frac{\text{cycles}}{\text{sec}}\right)$	CHEMICAL SHIFT (UNSONICATED)(a) $\left(\frac{\text{cycles}}{\text{sec}}\right)$
2%	.40	.45
6%	.90	1.20
10%	1.55	1.70

(a) Shifts are relative to distilled water.

A method suggested by Reilly, McConnell, and Meiseonheimer (13) was used to measure differences in volume diamagnetic susceptibility of DNA-Water mixtures and water alone. In a coaxial tube arrangement, in the

absence of rotation, the liquid in the annulus between the two glass tubes gives rise to a broad resonance signal with two peaks, owing to the fact that the field experienced by the molecules in the annulus is not uniform. The separation of the two peaks, ΔH, relative to the applied field H_0 is given by

$$\frac{\Delta H}{H_0} = 4\pi\left[(x_1-x_2)\frac{a^2}{r^2} + (x_2-x_3)\frac{b^2}{r^2}\right]$$, where x_1, x_2, and x_3 are the volume diamagnetic susceptibilities of the liquid in the inner glass tube, the glass tube, and the annular liquid, respectively. The radius r refers to the mean radius of the annular liquid, and a and b are the inner and outer radii of the inner glass tube. By measuring the separation between peaks, the volume diamagnetic susceptibilities can be found.

When an external reference is used in measuring the "chemical shift", a correction involving the difference in bulk diamagnetic susceptibility between the external reference and the sample must be applied. This is necessitated by the fact that, in the cylindrically shaped containers, the actual fields experienced by individual nuclei will depend on the magnetic polarization near the surface.

In Plate V, Figure 2, "chemical shift" corrections due to differences in volume diamagnetic susceptibility between water in DNA mixtures and free water are shown as a function of concentration of DNA. Equation 4.6 of Pople, Schneider, and Bernstein (14) was used in calculating "chemical shift" corrections from volume diamagnetic susceptibility values. The difference is linear with respect to concentration. When the corrections due to diamagnetic susceptibility are made to the

"chemical shift" of various DNA-water mixtures, the corrected "chemical shifts" correspond to that of free water. The linear relationship of bulk diamagnetic susceptibility versus concentration of DNA corresponds to Weidemann's additivity law (15) for molecular mixtures, $x = x_1p_1 + x_2p_2 + \ldots x_np_n$ where x is the susceptibility of the mixture; x_n, p_n are susceptibilities and weight fractions respectively for the components.

Because bound water was not detected by the previous measurements, studies were then made to determine the effect of water structure on the denaturation temperature of DNA in solution by proton resonance techniques.

The "chemical shift" was measured for various DNA concentrations at high temperatures to determine the effect of denaturation of calf thymus DNA on the magnetic environment of the water proton. A Varian V-6057 Variable Temperature System with a High Temperature Control was used for this purpose. Resonance spectra were taken in the temperature interval from 45° C to 95° C. A time interval of approximately ten minutes was alloted between each temperature run to allow the sample to reach an equilibrium temperature. The CH_2 resonance peak of ethylene glycol was used as an external reference for all measurements. Ethylene glycol was placed in a thin cylindrical capillary tube inside the sample. Due to the coaxial tube arrangement, a correction due to the difference in bulk diamagnetic susceptibility between the external reference and the sample was necessary in determining exact magnetic field positions. The "chemical shift" was found by measuring the distance between the resonance peak corresponding to the CH_2 group of ethylene glycol and

the resonance peak for the sample. The CH_2 resonance peak of ethylene glycol was positioned at a higher magnetic field than the resonance peaks due to the water mixtures. All distances between resonance peaks were measured in units of frequency. Larger frequency separations corresponded to lower magnetic field positions for the proton resonance peaks of the various water mixtures. Three separate runs were made at each temperature interval and the distance between resonance peaks was averaged for the three runs.

In Plate VI, the "chemical shift" is plotted versus temperature for a .15 M NaCl, 1% sodium citrate solution and for water. The magnetic resonance peak due to water is moved to a higher magnetic field position in the presence of .15 M NaCl, 1% sodium citrate. However the effect of .15 M NaCl, 1% sodium citrate on the "chemical shift" of water is less at high temperatures than at 45° C. The "chemical shift" is approximately linear with increase in temperature for both the .15 M NaCl, 1% sodium citrate solution and for water.

The "chemical shift" was measured as a function of temperature for different concentrations of DNA in a .15 M NaCl, 1% sodium citrate solution. The results of these measurements for 2% DNA and 8% DNA are shown in Plate VII. Except for the differences in bulk diamagnetic susceptibility for the various samples, the "chemical shift" of the DNA solutions are practically identical to the .15 M NaCl, 1% sodium citrate solution.

The dependence of the solvent on the "chemical shift" of DNA solutions was also verified for DNA containing no salt. The "chemical shift" of DNA containing no salt was practically identical to that for water at different temperatures. No difference in the "chemical shift" with

EXPLANATION OF PLATE VI

"Chemical shift" of a .15 M NaCl, 1% sodium citrate solution, and water at different temperatures. (A), water; (B), .15 M NaCl, 1% sodium citrate.

PLATE VI

CHEMICAL SHIFT (CYCLES/SEC)
60
55
50
45
40
35
40
50
60
70
80
90
100
TEMPERATURE (°C)
(B)
(A)

EXPLANATION OF PLATE VII

"Chemical shift" of two different DNA-water mixtures and a .15 M NaCl, 1% sodium citrate solution at different temperatures. (A), .15 M NaCl, 1% sodium citrate; (B), 2% DNA, .15 M NaCl, 1% sodium citrate; (C), 8% DNA, .15 M NaCl, 1% sodium citrate.

PLATE VII

CHEMICAL SHIFT (CYCLE/SEC)
65
60
55
50
45
40
35
40
50
60
70
80
90
100
TEMPERATURE
(°C)
(A)
(B)
(C)

increase in temperature could be observed for DNA solutions which had been previously denatured from samples which had not been previously denatured.

Because of the powerful denaturing effect of concentrated solutions of several salts on DNA, the "chemical shift" was measured for different concentrations of $NaClO_4$ in 6% DNA solution and in water. Concentrated solutions of $NaClO_4$ are known to lower the thermal denaturation temperature of DNA at neutral pH by as much as 60° C. (16) The effect is believed to be due mainly to the ClO_4^- ion. It is believed that the ClO_4^- ion is not, in the usual sense, a hydrogen bond breaking agent but that the anion exerts its effect on DNA largely through it's modification of the structure of water. (16) If this is true, it could be classified as a hydrophobic bond breaking agent.

The results of the "chemical shift" versus temperature measurements for different $NaClO_4$ solutions are shown in Plate VIII. The shifts are approximately linear with respect to molar concentration up to 4.1 M $NaClO_4$. Above 4.1 M $NaClO_4$, the concentration dependence of the shift becomes markedly smaller. Shifts are to high magnetic field positions in the presence of $NaClO_4$ which would correspond to a "more disordered" water structure. The $NaClO_4$ solutions exert practically as much effect on the "chemical shift" of a water solution at high temperatures as at low temperatures. The "chemical shift" for different concentrations of $NaClO_4$ in 6% DNA solution behave identically to the "chemical shift" for the different $NaClO_4$ concentrations alone. The results of these measurements can be seen in Plate IX. The resonances for different $NaClO_4$ concentrations in DNA solutions are displaced slightly to lower magnetic

EXPLANATION OF PLATE VIII

"Chemical shift" of various $NaClO_4$ concentrations at different temperatures. (A), 1% sodium citrate; (B), .97 M $NaClO_4$, 1% sodium citrate; (C), 2.7 M $NaClO_4$, 1% sodium citrate; (D), 4.1 M $NaClO_4$, 1% sodium citrate; (E), 5.2 M $NaClO_4$, 1% sodium citrate.

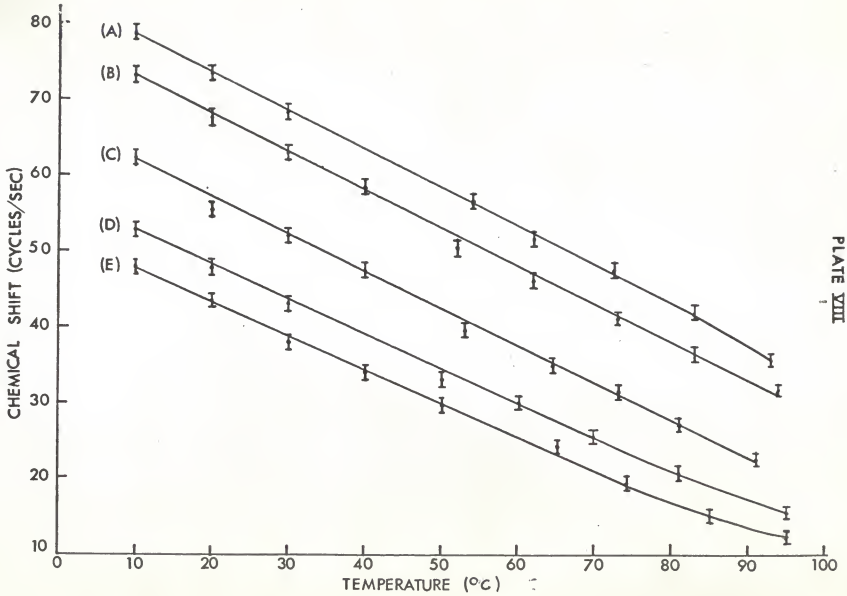

(A)
(B)
(C)
(D)
(E)
CHEMICAL SHIFT (CYCLES/SEC)
TEMPERATURE (°C)
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100

PLATE XIII

EXPLANATION OF PLATE IX

"Chemical shift" of various $NaClO_4$ concentrations in 6% DNA solution at different temperatures. (A), 6% DNA, 1% sodium citrate; (B), 6% DNA, .97 M $NaClO_4$, 1% sodium citrate; (C), 6% DNA 2.7 M $NaClO_4$, 1% sodium citrate; (D), 6% DNA 4.1 M $NaClO_4$, 1% sodium citrate, 1% sodium citrate; (E), 6% DNA, 5.2 M $NaClO_4$, 1% sodium citrate.

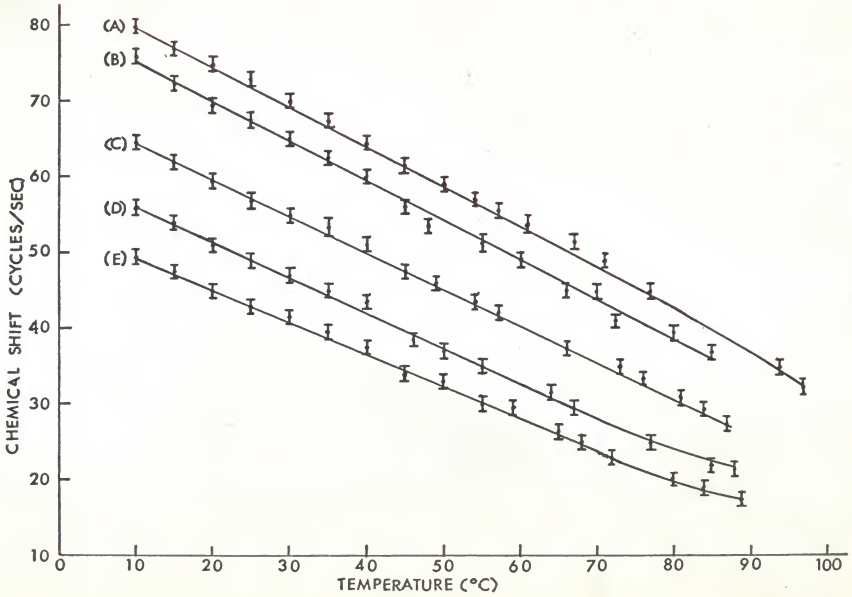

(A)
(B)
(C)
(D)
(E)
CHEMICAL SHIFT (CYCLES/SEC)
TEMPERATURE (°C)
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100

PLATE IX

field positions due to the difference in bulk diamagnetic susceptibility between the DNA solution and the salt solution.

"Chemical shifts" were also measured for different concentrations of $NaClO_4$ with a 2.9 M NaCl concentration added. In Plate X the results of these measurements are shown. At low temperatures, resonance peaks are displaced to higher magnetic field positions due to the presence of NaCl while at high temperatures, resonance peaks are displaced to lower field positions.

It is believed that the chloride anion has no marked structure breaking effect. (17) The effect of anion hydration on the structure of water is the subject of some debate. Both Brady (18) and Hindman (11) interpret their results to indicate that the anions are not hydrated in the same sense as cations. The negative ions have a larger effect on the proton resonance than the positive ions. The explanation is believed to be in the fact that the proton in a hydration shell around a negative ion is near the negative ion and is directly influenced by it, while in the hydration shell around a positive ion the field the proton experiences is screened by the electron pair of the oxygen. (10) Relative molar shifts of the proton resonance for different ions were calculated by Schoolery and Alder. (10) They obtained values of $\delta = -.85$ for ClO_4^-, $\delta = -.57$ for Na^+, and $\delta = -.01$ for Cl^-, where δ is defined as

$$\delta = \frac{(H_{H_2O} - H_{Sample})}{H_{H_2O}} \times 10^7 \quad .$$

Half-widths were approximately the same for all concentrations of $NaClO_4$ in 6% DNA. Half-widths of the resonance spectra decreased regularly from 10° C to 40° C and remained at a constant value from 40° C to 85° C.

EXPLANATION OF PLATE X

"Chemical shift" of various $NaClO_4$ concentrations in 2.9 M NaCl at different temperatures. (A), 2.9 M NaCl; (B), 2.9 M NaCl, .93 M $NaClO_4$; (C), 2.9 M NaCl, 2.56 M $NaClO_4$; (D), 2.9 M NaCl, 3.84 M $NaClO_4$; (E), 2.9 M NaCl, 4.96 M $NaClO_4$.

PLATE X

CHEMICAL SHIFT (CYCLES/SEC)
70
60
50
40
30
20
10
20
30
40
50
60
70
80
90
100
TEMPERATURE (°C)
(A)
(B)
(C)
(D)
(E)

No correlation could be made between the half-widths of the water proton p resonance spectra and the "structure temperature" of water in DNA sodium perchlorate solutions.

In order to determine a relationship between the modification of the water structure due to $NaClO_4$ and the denaturation temperatures of DNA in the presence of concentrated solutions of $NaClO_4$, ultraviolet absorption measurements on DNA solutions with different concentrations of $NaClO_4$ and NaCl were made.

Absorbance measurements were made at 260 mu with a Zeiss spectrophotometer, Model PMQ II, with a Haake attachment for thermostatic control of the temperature in the block in which the cuvettes were housed. Glass-stoppered 3.0 ml silica cuvettes with a light path of 1 cm were used. Absorbance measurements were made at 2° C intervals with 10 minutes being allotted at each temperature to allow for the establishment of thermal equilibrium. The DNA used was at a concentration of approximately 50 $\frac{\mu g.}{ml}$.

The temperature, T_i, at which denaturation commences for different DNA solutions and the temperature, $T_{\frac{1}{2},d}$, at which the increase in optical density is 50% of it's maximum value obtained upon denaturation, were measured for the DNA solutions in the presence of electrolytes. The results of these measurements can be seen in Table V. Large concentrations of $NaClO_4$ tended to lower the denaturation temperatures of DNA solutions considerably. Large sodium concentrations were used to decrease electrostatic repulsions between imperfectly shielded phosphate groups so that denaturation temperatures would not be dependent upon the electrostatic shielding of the phosphate groups.

Table V. Initial Denaturation Temperatures and Thermal Denaturation Temperatures of DNA Solutions for Different Concentrations of $NaClO_4$ and NaCl.

CONCENTRATION OF $NaClO_4$ and NaCl IN DNA SOLUTIONS (MOLES)(a) (LITER)	T_i (°C)	$T_{i,d}$ (°C)
.15 M NaCl	78.0	86.3
.15 M NaCl .97 M $NaClO_4$	80.0	87.0
.15 M NaCl 2.70 M $NaClO_4$	72.0	78.8
.15 M NaCl 4.10 M $NaClO_4$	64.0	73.0
.15 M NaCl 5.20 M $NaClO_4$	52.0	63.3
1 M NaCl	85.5	92.0
1 M NaCl .96 M $NaClO_4$	84.0	88.8
1 M NaCl 2.67 M $NaClO_4$	72.0	80.3
1 M NaCl 4.00 M $NaClO_4$	63.0	71.7
1 M NaCl 5.15 M $NaClO_4$	52.0	62.2
2.9 M NaCl	86.0	95.0
2.9 M NaCl .93 M $NaClO_4$	78.0	87.2
2.9 M NaCl 2.56 M $NaClO_4$	67.0	76.0
2.9 M NaCl 3.84 M $NaClO_4$	58.0	68.7
2.9 M NaCl 4.96 M $NaClO_4$	50.0	60.0
4.5 M NaCl	81.0	86.7
4.5 M NaCl .88 M $NaClO_4$	76.0	85.0

(a) The solvent used was .015 M sodium citrate.

Ultraviolet absorption measurements were also made on 6% DNA solutions using a brass cell consisting of quartz windows separated by foil of thickness 1 mil. DNA was inserted in the space between the two quartz windows of thickness 1 mil. Optical density values of 1 were obtained by using a foil of this thickness. Increases in optical density of approximately 50% were observed in the temperature interval from 38° C to 55° C. In all cases, the increase in optical density occurred at a much lower temperature for the highly concentrated DNA solutions than for the more dilute DNA solutions.

Because of the different kinds of effects of anions and cations on the structure of water, (10, 11) the shift of $NaClO_4$ was separated into relative molar shifts due to the sodium and perchlorate ions respectively. Values calculated by Schoolery and Alder (10), which are in agreement with those of Hindman (11), were used in evaluating relative molar shifts. The calculated shift of the ClO_4^- ion was found to be 60% of the shift for sodium perchlorate. Because of the small change in "chemical shift" of $NaClO_4$ with respect to water at different temperatures, the shift due to the ClO_4^- ion was assumed to be independent of temperature. The effect of the ClO_4^- ion on the "chemical shift" versus temperature curves for different $NaClO_4$ concentrations can be seen in Plate XI.

By identifying the "chemical shifts" due to the ClO_4^- ion corresponding to the initial denaturation temperatures of the highest $NaClO_4$ concentrations in DNA at constant NaCl concentrations, temperatures where other concentrations of $NaClO_4$ have the same "chemical shift" can be found by drawing a horizontal line corresponding to a fixed value of "chemical shift" through the "chemical shift" versus temperature curves. Bernal

EXPLANATION OF PLATE XI

"Chemical shift" due to the ClO_4^- ion for various $NaClO_4$ concentrations as a function of temperature. (A), 1% sodium citrate; (B), .97 M $NaClO_4$, 1% sodium citrate; (C), 2.7 M $NaClO_4$, 1% sodium citrate; (D), 4.1 M $NaClO_4$, 1% sodium citrate; (E), 5.2 M $NaClO_4$, 1% sodium citrate.

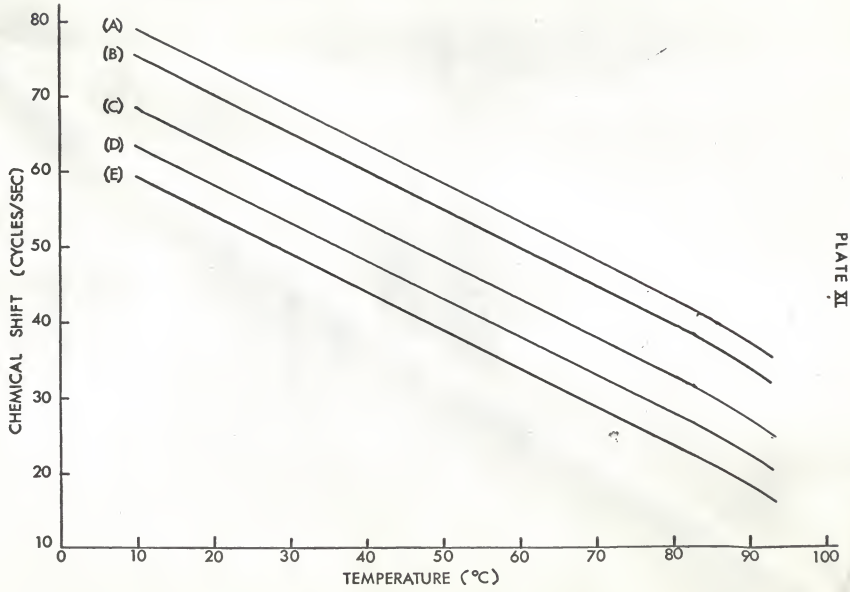

(A)
(B)
(C)
(D)
(E)
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100
CHEMICAL SHIFT (CYCLES/SEC)
TEMPERATURE (°C)

PLATE XI

and Fowler (19) suggested that water has a broken-down ice structure which still maintains most of the hydrogen bonds and that a gradual decrease in hydrogen bonding corresponds to increased temperature. Using this concept, a constant "chemical shift", due to the ClO_4^- ion, would correspond to a constant water "structure temperature".

The measured temperatures corresponding to a constant "chemical shift" due to the ClO_4^- ion and the initial denaturation temperatures of DNA for different $NaClO_4$ and NaCl concentrations are shown in Plate XII, Figures 1 and 2, and Plate XIII. For high sodium concentrations, there was a close relationship between the initial denaturation temperatures of DNA in different $NaClO_4$ solutions and a constant "chemical shift" due to the ClO_4^- ion. The difference in electrostatic shielding of the phosphate groups should be most pronounced for low concentrations of $NaClO_4$ and NaCl. This probably explains the lack of correlation between constant "chemical shifts" due to the ClO_4^- ion and initial denaturation temperatures for the .15 M NaCl solutions at low $NaClO_4$ concentrations.

Relationships between thermal denaturation temperatures of DNA in different $NaClO_4$ solutions and constant "chemical shifts" due to the ClO_4^- ion were obtained also. However in most cases, initial denaturation temperatures fitted constant "chemical shift" values slightly better than thermal denaturation temperatures.

No correlation between initial denaturation temperatures of DNA in the presence of electrolytes and constant "chemical shifts" due to both the Na^+ ion and the ClO_4^- ion could be obtained. The observation is in agreement with Hindman's results on the sodium and perchlorate ions; the chemical shift of the sodium ion is predominantly due to hydration while

EXPLANATION OF PLATE XII

Fig. 1. Initial denaturation temperatures of DNA and constant "chemical shift" temperatures due to the ClO_4^- ion for different $NaClO_4$ concentrations in a .15 M NaCl solution. ⊙, initial denaturation temperatures of DNA; ⊗, constant "chemical shift" temperatures.

Fig. 2. Initial denaturation temperatures of DNA and constant "chemical shift" temperatures due to the ClO_4^- ion for different $NaClO_4$ concentrations in a 1 M NaCl solution. ⊙, initial denaturation temperatures of DNA; ⊗, constant "chemical shift" temperatures.

PLATE XII

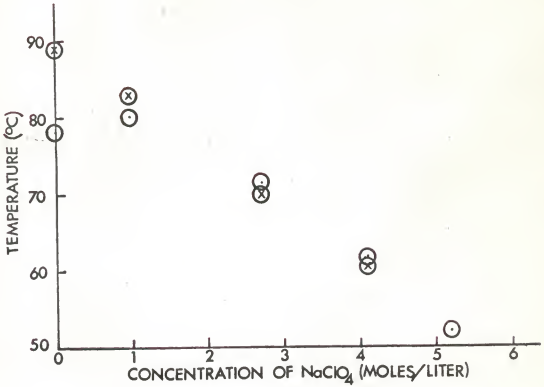


Fig. 1

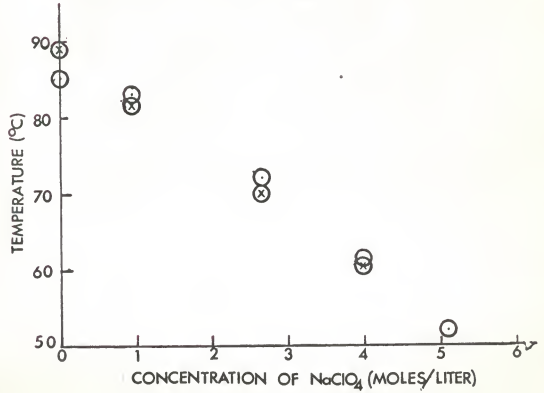


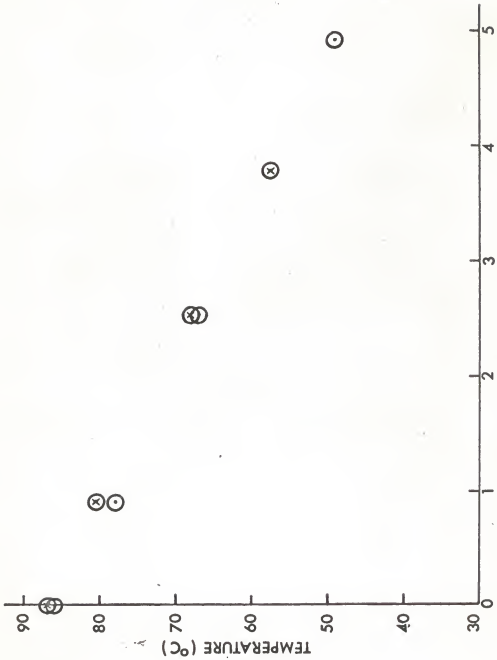
Fig. 2

EXPLANATION OF PLATE XIII

Initial denaturation temperatures of DNA and constant "chemical shift" temperatures due to the ClO_4^- ion for different $NaClO_4$ concentrations in a 2.9 M NaCl solution.

⊗ , initial denaturation temperatures of DNA;
⊗ , constant "chemical shift" temperatures.

PLATE XIII


TEMPERATURE (°C)
90
80
70
60
50
40
30
0
1
2
3
4
5
CONCENTRATION OF NaClO4 (MOLES/LITER)

the "chemical shift" of the perchlorate ion is predominantly due to what Hindman calls the "structure factor" effect.

In order to determine the effect of D_2O on denaturation temperatures of DNA in the presence of electrolytes, ultraviolet absorption measurements of DNA were made for various concentrations of $NaClO_4$ in a 1 M NaCl, .015 M sodium citrate, 90% D_2O solutions. Previous studies of bacterial DNA denaturation in H_2O, H_2O-D_2O, and D_2O citrate-saline solutions have indicated no difference in the thermal denaturation temperature of DNA in D_2O solutions as compared to H_2O solutions. (20)

In Table VI are shown comparisons of initial denaturation temperatures and thermal denaturation temperatures for different $NaClO_4$ concentrations of DNA in 1 M NaCl, .015 M sodium citrate, 90% D_2O and 1 M NaCl, .015 M sodium citrate, H_2O solutions. Both, initial denaturation

Table VI. Initial Denaturation Temperatures and Thermal Denaturation Temperatures of DNA Solutions for Different Concentrations of $NaClO_4$ and NaCl in Two Different Solvents.

CONCENTRATION OF $NaClO_4$ AND NaCl in DNA SOLUTIONS (MOLES) (LITER)	T_i 90% D_2O (Solvent) (°C)	T_i H_2O (Solvent) (°C)	$T_{\frac{1}{2}}$,d 90% D_2O (Solvent) (°C)	$T_{\frac{1}{2}}$,d H_2O (Solvent) (°C)
1 M NaCl	85.0	85.0	92.8	92.0
1 M NaCl .96 M $NaClO_4$	82.0	84.0	89.0	88.8
1 M NaCl 2.67 M $NaClO_4$	70.0	72.0	78.8	80.3
1 M NaCl 4.00 M $NaClO_4$	62.0	63.0	72.7	71.7
1 M NaCl 5.15 M $NaClO_4$	52.0	52.0	63.0	62.2

temperatures and thermal denaturation temperatures were practically identical for DNA in the two different solvents.

In determining the effect of the 90% D_2O solution on the proton magnetic resonance signal, "chemical shifts" were measured at different temperatures for a 90% D_2O, .015 M sodium citrate solution. A comparison of the measurements with the shifts for .015 M sodium citrate is shown in Plate XIV. Small shifts upfield were observed for 90% D_2O solutions as compared to H_2O. At 37° C, the shift upfield was 3 $\frac{\text{cycle}}{\text{sec}}$, while at 85° C, the shift was 4.5 $\frac{\text{cycle}}{\text{sec}}$ with respect to water. By using the method suggested by Reilly, McConnell, and Meisenheimer (13) for measuring differences in volume diamagnetic susceptibility for 90% D_2O and H_2O, a correction of 2.5 $\frac{\text{cycle}}{\text{sec}}$ due to the difference in susceptibility of the two solvents was obtained at 37° C. The corrected "chemical shift" of 90% D_2O, .015 M sodium citrate therefore obtained was .5 $\frac{\text{cycle}}{\text{sec}}$ (corresponding to $\Delta T \equiv 1°$ C) upfield with respect to water at 37° C. Proton magnetic resonance shifts in H_2O - D_2O mixtures have been studied previously. (21) Proton magnetic resonance shifts linear with respect to deuterium concentration were obtained in these studies.

Chemical shifts were measured for different $NaClO_4$ concentrations in a 1 M NaCl, .015 M sodium citrate, 90% D_2O solution at several temperatures. The results of these measurements are shown in Plate XV. The concentration dependence of the shift becomes smaller for $NaClO_4$ concentrations above .96 M.

The method outlined previously was used in comparing initial denaturation temperatures of DNA in 90% D_2O solutions for different $NaClO_4$ concentrations with the shift due to the ClO_4^- ion in a 90% D_2O solution. In

EXPLANATION OF PLATE XIV

"Chemical shift" of 90% D_2O - 10% H_2O and 100% H_2O at different temperatures.
(A), 100% H_2O; (B), 90% D_2O - 10% H_2O.

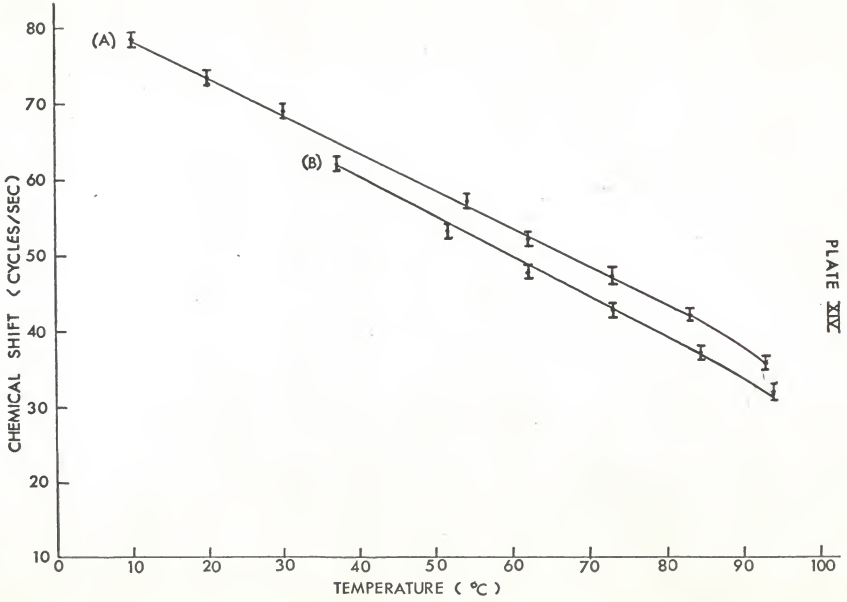

(A)
(B)
CHEMICAL SHIFT (CYCLES/SEC)
TEMPERATURE (°C)
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90
100

PLATE XIV

EXPLANATION OF PLATE XV

"Chemical shift" of various $NaClO_4$ concentrations in 90% D_2O, 1 M NaCl at different temperatures. (A), 90% D_2O, 1 M NaCl; (B), 90% D_2O, 1 M NaCl, .96 M $NaClO_4$; (C), 90% D_2O, 1 M NaCl, 2.67 M $NaClO_4$; (E), 90% D_2O, 1 M NaCl, 5.15 M $NaClO_4$; (D), 90% D_2O, 1 M NaCl, 4 M $NaClO_4$.

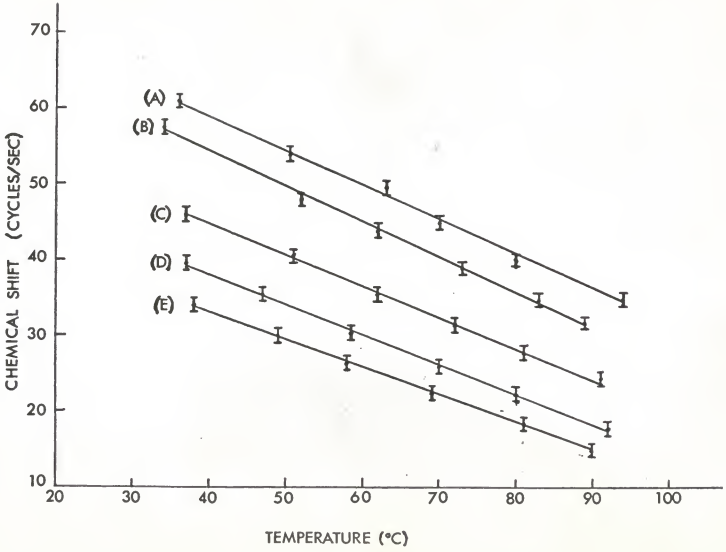

70
60
50
40
30
20
10
20
30
40
50
60
70
80
90
100
(A)
(B)
(C)
(D)
(E)
CHEMICAL SHIFT (CYCLES/SEC)
TEMPERATURE (°C)

PLATE XV

Plate XVI, initial denaturation temperatures and temperatures corresponding to a constant "chemical shift" for the ClO_4^- ion are shown at different concentrations of $NaClO_4$. Close relationships were again obtained between the initial denaturation temperatures of DNA and constant shift values for different ClO_4^- concentrations in the 90% D_2O solution.

CONCLUSIONS

I. Effects of the DNA molecule on water.

The results of area measurements obtained from proton magnetic resonance studies of water in DNA mixtures indicate that nearly all the water protons in DNA mixtures contribute to the NMR signal. Under these conditions, only a small fraction of the water in such a solution exists in a bound, lattice-ordered form. From self-diffusion studies of water in DNA, Wang (22) calculated that .35 grams of water per gram of dry sodium desoxyribonucleate is considered to be hydrated. Due to the ±5% uncertainty in area measurements, a decrease in area of the magnitude associated with hydrated water could not have been distinguished in the present experiments.

Line broadening of water in DNA mixtures is probably due to either proton exchange between water and protons of DNA or exchange between free and bound water protons. In both cases, half-widths should increase as a function of DNA concentration. This was observed in the present experiments. Spin exchange between protons causes a decrease in T_2, the spin-spin relaxation time. Balazs, Bothner-By, and Gergely (7) measured no appreciable difference in T_1 for water in DNA mixtures as compared to T_1 for water. By assuming T_1 constant for 1.6% DNA and for water, the decrease in the product T_1T_2 observed for 1.6% DNA must be due to a

EXPLANATION OF PLATE XVI

Initial denaturation temperatures of DNA and constant "chemical shift" temperatures due to the ClO_4^- ion for different $NaClO_4$ concentrations in a 90% D_2O, 1 M NaCl solution.

⊙ , initial denaturation temperatures of DNA;

⊛ , constant "chemical shift" temperatures.

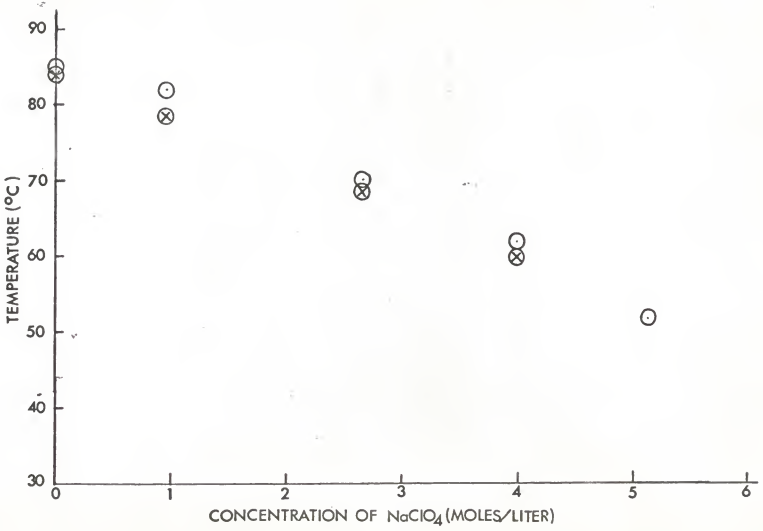

90
80
70
60
50
40
30
TEMPERATURE (°C)
0
1
2
3
4
5
6
CONCENTRATION OF $NaClO_4$ (MOLES/LITER)

PLATE XXI

decrease in T_2. This contradicts the theory that long-range inhomogeneities due to the magnetic anisotropy of the DNA molecules produce a broadening of the NMR signal because under those conditions, T_2 could remain the same for water in DNA mixtures as compared to water alone.

One might expect the π-electrons contained in parallel layers of the purine and pyrimidine bases to influence the magnetic environment of water in DNA mixtures, but since no change is observed in the corrected "chemical shifts" of DNA-water mixtures as compared to water at different temperatures, the π-electrons seem to have little or no effect on water in DNA before or after denaturation.

The small decrease in line width of sonicated DNA samples would seem to denote a decrease in viscosity due to lower molecular weights. However, previous studies of denaturation of DNA by shearing forces (12) indicate that highly concentrated DNA solutions afford each other mutual protection against degradation by shear forces and that sonication should have little or no effect on highly concentrated solutions.

II. The influence of water on denaturation of DNA.

The close relationship obtained between initial denaturation temperatures of DNA in $NaClO_4$ solutions and the "chemical shift" due to the ClO_4^- ion indicate that initial denaturation temperatures of DNA depend on the amount and kind of ordering of the water structure in DNA solutions. If one considers the effect of both the Na^+ ion and the ClO_4^- ion on the "chemical shift" due to $NaClO_4$, no such relationship can be obtained. This may be due to cations and anions affecting water structure differently. Hamaguchi and Geiduschek (16) observed only minor differences in effect

of the cations Li^+, Na^+, and K^+ on helix stability. Among the anions, on the other hand, great variations of denaturing power are observed. Near-infrared studies of the structure of water in ionic solutions indicate that the perchlorate ion has a very large order-destroying effect on water clusters while the amount of anion hydration and of ion-pair formation is not very large. (17)

The substitution of large quantities of H_2O by D_2O in DNA solutions further supported the hypothesis that the ordering of the water structure has an effect on initial denaturation temperatures of DNA. Reports on the biological effects of heavy water indicate that processes involved in cell division are markedly affected by substitution of H_2O by D_2O. It has been suggested that this effect of D_2O may be due to changes in the strength of hydrogen bonds involved in helix-coil transitions of proteins and nucleic acids. (23) If the strength of hydrogen bonds involved in the helix-coil transition for DNA are changed on substitution of H_2O by D_2O, then one would expect a change in the thermal denaturation temperature of DNA solutions, provided intramolecular hydrogen bonds between base pairs provide a large fraction of the energy required for DNA helix stabilization. However no change in thermal denaturation temperatures of DNA in D_2O solutions have been observed in the present experiments or in previous studies. (20) The possibility arises that D_2O substitution for H_2O might influence the strength of hydrogen bonds involved in helix-coil transitions for proteins and DNA differently. If the strength of hydrogen bonds involved in the helix-coil transition of DNA is changed very little by substitution of H_2O by D_2O, then one could not discount the effect of intramolecular hydrogen bonds between base pairs as providing a fraction of the energy required

for stabilization of the DNA helix.

Present studies have provided experimental evidence that hydrophobic forces supply a large fraction of the energy required for helix stabilisation of DNA in aqueous solutions. However for a more concise analysis of hydrophobic bonding and it's effect on the stabilization of the DNA helix, the structure of water and the effects of electrolytes on water structure at different temperatures must be studied more thoroughly.

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A STUDY OF THE INFLUENCE OF WATER ON THE DENATURATION OF DEOXYRIBOSE NUCLEIC ACID

by

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The effects of DNA on aqueous solutions has been studied. Proton magnetic resonance techniques were used for these measurements. From the results of area measurements; no hydrated water (water bound to DNA) nor ice-like structures of water in DNA could be detected. Due to a 5% uncertainty in area measurements, a 5% decrease in the number of water protons contributing to the absorption curve could not have been detected. No effect due to the diamagnetic anisotropy produced by the stacked planar arrangement of purine and pyrimidine bases in DNA could be seen.

Proton magnetic resonance studies of electrolyte solutions were made at different temperatures. Because of the structure breaking ability of the ClO_4^- ion on water, chemical shifts due to the perchlorate ion in $NaClO_4$ solutions were compared with initial denaturation temperatures of DNA in the same solutions. For high sodium concentrations, a close relationship was obtained between constant "chemical shifts" due to the ClO_4^- ion and initial denaturation temperatures of DNA in sodium perchlorate solutions. The relationship also held for $NaClO_4$ concentrations in a 90% D_2O - 10% H_2O solution. On the basis of these experiments, the following proposal can be set forth and discussed; due to the ClO_4^- ion having the same effect on water temperature as an increase in water temperature, "water structure temperature" has a large effect on temperatures at which DNA denatures. In this case, hydrophobic forces must provide a large fraction of the stabilization energy required for the double-helical structure of DNA. To further enhance the study of the effects of water structure on stabilization of the double-helical form of DNA, the effects of electrolytes on water structure at different temperatures must be studied more thoroughly.