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EFFECTS OF RADIOFREQUENCY RADIATIONS ON SELECTED BIOMOLECULES IN RESPONSE AND COUPLING WITH THE EFFECT OF RADIATION FROM MICROWAVE SOURCE

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ABSTRACT

The increasing use of radio frequencies to operate systems remotely has led to concern over changes that may be taking place in biological systems, namely the human population. The investigation proposed will be one in which a wide range of radio and microwave frequencies are employed to test for response of biological molecules to such radiation. The hyaluronic molecule and select DNA will be studied for response and coupling with the radiation from microwave sources. In this laboratory we have radiofrequency generators that cover the range of a few MHz to 40 GHz along with computers and other ancillary devices needed to conduct and analyze the results of an investigation. This research will bring together an expert in the field of Biology and experts in the field of Physics to study this significant research. It is proposed that this collaboration will be a "win-win" situation for India and America in that knowledge will be gained on a very important aspect of human health.

INTRODUCTION

The hyaluronic acid molecule is a long chain proteoglycan with both interesting and chemical properties. Because of the important role that this molecule plays in the human body it is worthy of study.

Jensen and Villstrup [Dato] first noted that samples of hyaluronic acid HA had the ability to behave as piezzo-electric devices, developing about 100 mV of potential when subject to bending stress. A model was even proposed that an aggregate of such molecules could act as charged cylinders which could "float on each other to form an array with only minimum friction. The ability of the HA molecules to act as lubricants in body joints make it an interesting and exciting candidate for study in any range of the electromagnetic spectrum.

Some microwave properties of this molecule were studies for temperature [Dahiya, 1981] and pH [Jani, 1980] of its environment effects to try to better understand what role either and or temperature and pH had on molecular function.

This proposal is to conduct a series of controlled experiments over the range of frequencies from 8.2 GHz to 25 GHz, as such frequencies, as well as others, are prevalent in the environment. This range of frequencies was chosen because the project director and co-project director are already capable of using tuned resonant microwave cavities to study the properties of matter over the range. Moreover, the size of the resonant cavities is convenient for studying this range of microwave frequencies. The cavity has already been designed and tested for the work, but the range of study will be broadened as dictated if future studies, toward lower frequencies as our expertise develops and such results are obtained to dictate the cost and most effective range of frequencies to cover.

Inconclusive data and results have appeared in the literature (references) about the effects of telephone signal carriers, 800 MHz of 1200 MHz, we will investigate the feasibility of adapting our studies to the range of 500 MHz to 8,200 MHz, as time permits, Initially, however, we will confine our attention to the range of frequencies, 8.2 GHz to 25 GHz, wherein we both expertise and most of the equipment has already been developed.

Since hyaluronic acid is a biomacromolecule, one might expect it to exhibit a unique temperature-dependent behavior with relaxation mechanism near physiological temperatures. The conformational changes in the hyaluronic acid molecule in various environments will be observed by monitoring its dielectric response at microwave frequencies subjected to specific incurments of temperatures buffer pH and select molecular incurments.

A tunable resonant microwave cavity operated in the TE 011 mode in a frequency range of 8.2 to 12.4 GHz will be used to measure the dielectric response of the hyaluronate solutions. Both the real and imaginary parts of the complex dielectric constant will be obtained by using the perturbation equation analysis of the dielectric response of specimens loading the recent microwave cavity. The dielectric constants can be related to effective dipole moments and to molecular conformation and to the extent of aggregation of the hyaluronic acid molecules into super molecular structures. In addition to the dielectric response, relaxation times can be used to discuss the extent of ordering of solute by the solvent at the molecular level.

The Dielectric properties and relaxation times are generally sensitive to temperature changes. Experiments will be performed at different temperatures, especially those of physiological importance. Energies of activation and the more rigorous enthalpies and entropies of transition for conformational changes and for relaxation processes can be determined from measurements at different temperatures as the samples act as a load in the resonant microwave cavity.

The structural information obtainable from the proposed dielectric properties experiments is indirect and lacking in some fine detail lacking in some fine detail, theoretical calculations of the conformational statistics will generate a Boltzmann distribution of conformations which can be used to calculate molecular properties such as dipole moments, heat capacities, and partition

functions. These properties can be related to experimental results so that differences in experimental behavior can be related to changes in conformation at a fundamental molecular level.

The dielectric behavior of hyaluronic acid at a number of microwave frequencies will reveal important information about the electrical characteristics of this molecule. The outcome of such experiments will then be used to devise suitable experiments to conduct in vivo tests on humans to elaborate the effects of electromagnetic waves on biological systems, specifically the hyaluronic acid molecule found in humans.

Any biolayered molecule that can serve as an agent to test for microwave interaction is a good one but the fact that hyaluronic acid has already been shown to respond to microwaves indicates it to be promising molecule to start with. The dielectric behavior of the hyaluronic acid taken from vitreous humor will reveal important information about the electrical properties of that molecule. The problem of retinal detachment is related to the characteristics of vitreous humor and a comparison of the dielectric studies of normal and abnormal samples of this molecule will be significant in looking into this problem more closely.

BACKGROUND AND SIGNIFICANCE

The macromolecules of biological tissues and fluids in health and diseases have attracted wide interest among scientists mainly because it is appreciated that knowledge of their structure, their mode of biosynthesis, and the ways in which they change or break down may lead to a better understanding of their function in the complex cycles within living systems. A study of the conformational changes of a biomacromolecule due to changes in its environment has become important. Biological processes that involve polysaccharides and oligosaccharides include cell binding, cell recognition, blood group typing, and prevention of ice nucleation in the blood of Antarctic fish. Some of these processes are better understood than others. The common feature among them is that they are based on saccharide composition and structure in a solvent environment.

The physiological functions of a biomacromolecule seem to be closely related to its molecular conformations. The knowledge of any conformational changes due to changes in its environment may lead to a proper understanding of its functions. Hyaluronic acid, a biomacromolecule with a usually high molecular weight and some other very important biological functions, is the subject of this investigation. Hyaluronic acid is present in the connective tissues of most vertebrates. Its function seems to be that of binding water in interstitial spaces and to hold cells together in jelly-like mass.

Hyaluronic acid has a molecular weight of several million. Each molecule consists of about 1000 repeating disaccharide units D-glucuronic acid linked to N-acetyl-D-glucosamine as shown in the following illustration. The disaccharides are linked in a manner.

Figure X Acidic properties are the result of ionization of the carboxylic acid group in the glucuronic acid. The degree of ionization of the acid will be affected by the pH of the solvent. Changes in ionic strength of the solvent will affect the stability of chain conformations through electrostatic interactions among the solvent's ions and chain carboxylates and among the chain carboxylates and other polar groups on the polysaccharide chain.

The conformational changes in the hyaluronic acid molecules in various environments will be observed by monitoring its dielectric response at microwave frequencies.

The objective of the computational portion of this project is to discover the general rules governing the three dimensional folding of oligosaccharides and polysaccharides in solution by the theoretical calculation of the conformational statistics of these compounds using Monte Carlo calculations. This will explore the relationship of saccharide conformations to the different types of linkages and saccharide composition and to the different types of solvent environments.

The long range objective is to learn how saccharide structures relate to the biological functions of polysaccharides. In order to accomplish this aim, the conformations of the saccharides will be determined with particular attention paid to the interactions among the carbohydrate side chains and the solvent. The relative importance of hydrogen bonding, nonbonding repulsions, and coulombic interactions as determiners of conformation will be studied.

The dielectric properties of solids, liquids, and gases at various frequencies can be observed experimentally by using resent circuits and cavities. Resonant circuit methods have been used up to a frequency of 100 MHz, but these methods are not suitable beyond this frequency range because of energy losses into space. Various techniquest have been used to investigate the dielectric behavior of polar and non-polar liquids at low frequencies. The dielectric constant values of water was determined by capacitance measurements made at frequencies varying from 500 Hz to 50 KHz (Rusche and Good 1966).

During the late 1930's, investigations of the dielectric constant of water in the MHz region were begun in order to study the relaxation processes of aqueous media. The rapid development of radar technology and guided wave techniques during World War II made possible investigations in the GHz, or microwave region. Definitive measurements of the dielectric properties of water were made by a number of investigators (Collie et al. 1948; Saxton 1952; Grant et al. 1957).

The proposed method will involve the use of a microwave resonant cavity in the microwave spectrometer to study the dielectric relaxation in hyaluronic acid. Slater's perturbation equations

will be used to find the real and imaginary parts of the dielectric constant of the molecule under investigation. The dielectric relaxation phenomenon will be studies through the phase transition temperature of the molecule, and relaxation times will be calculated using Debye's equations. The relaxation times at different temperatures will be used to calculate the free energy of activation of the molecule.

Because of the low resistance in a resonant cavity, a large value for the quality factor, Q, can be obtained. This is in contrast to the resonant circuit which has a low value of Q. As the sample to be studies is placed in the cavity, it perturbs the electric or magnetic field in the cavity depending on what mode the cavity is in, and from this either the electric permittivity or magnetic permeability can be calculated. The cavity resonator technique is a standard technique and has been used by several workers. Chatterjee (1952) used a circular cavity resonating in the E mode with a capillary tube specimen and tuning plunger. Collie et at. (1948) made use of a circular cavity resonating in the H , mode with a capillary tube specimen. Kobayshi and Ogawa (1970) used a resonant cavity in the TE mode to measure the dielectric constant of Mn-Zn ferrite powder at a microwave frequency of 8.935 GHz. Hong and Roberts (1974) used a cylindrical cavity resonating near 10 GHz in the TM , to determine the dielectric properties of liquids and solids. Jani *et al.* (1980) studies the dielectric relaxation in hyaluronate solutions using a cylindrical resonating cavity. Dahiya et at (1981) used a similar cavity to study the dielectric properties of some polar molecules near their phase Transition temperature. (We need to find some references near the present.)

The change in dielectric behavior of polar molecules as they change from solid to liquid is of great interest. Thedielectric constant of the substance drops abruptly near phase transition temperatures. In some substances, like water and nitrobenzene, the drop is so rapid that it is very difficult to identify precisely the temperature of the phase change. The method is so sensitive that second order phase changes can be monitored.

To monitor rapid temperature changes, the microwave resonant cavity is interfaced to a microcomputer. A temperature probe is attached to the resonant cavity and is monitored by a microcomputer. The probe consists of a set of thermistors designed to produce a linear voltage response. The microcomputer is programmed to record the rapid changes in temperature as the molecule under investigation goes through the phase change. The number of measurements taken during the rapid phase change is a great improvement over earlier techniques in which the temperature of the phase change has to be estimated. The computerized cavity can be used for other temperature studies without changing the hardware of electronics. Only the software needed to be modified to fit the experiment prepared for hyaluronic acid.

PROGRESS REPORT/PRELIMINARY STUDIES

At the physics department at Southeast Missouri State University the principal investigator has established a microwave spectrometer capable of being operated in the entire x-band of frequencies. (8.2-12.4 GHz). A Varian V-58 klystron used with the proper power supply will provide the full range for the x-band. The spectrometer has been used to study the dielectric relaxation mechanism in a number of liquid crystals at different temperatures to test its sensitivity. The temperature control mechanism for the spectrometer is very sensitive and makes it a very important tool to study the dielectric behavior of different materials as a function of temperature. Dielectric behavior of a sample of barium titanate as it goes from para electric cubic phase to ferroelectric tetragonal phase was very successfully studies using this spectrometer. The temperature of the resonant cavity was then lowered to monitor the phase change of barium titanate as it went from tetragonal to orthorhombic and then to the rhombohedral phases, respectively. Dielectric behavior as a function of temperature has also been studied for strontium titanate and lead titanate zirconate crystals. The materials to be studies are inserted along the perpendicular axis of the microwave resonant cavity through a micrometer drive. The length of the sample is then adjusted to get maximum perturbation of the electric field in the cavity as evidenced by the signal on the oscilloscope and a reading of the position of the marker for the Q of the cavity and the center frequency of the klystron is taken. The temperature of the cavity is then varied and Q-change/frequency shift data taken at each temperature. A temperature probe attached to the microwave resonant cavity is interfaced to a microcomputer. The microcomputer allows temperature data to be taken very rapidly as a dielectric undergoes a phase change over a small temperature interval temperature quality factor Q data and frequency shift data are sent to three channels of a computer. A computer program utilizing Slater's perturbation equations then calculates the real and imaginary parts of the complex permittivity for the material under study.

During 1979-80 the principal investigator studies the dielectric behavior of a sample of hyaluronic acid taken from the human umbilical cord at a fixed microwave frequency of 9.0 GHz. This study was done at the microwave spectroscopy of North Texas State University now known as the University of North Texas. A tunable microwave resonant cavity was used as a probe in monitoring the dielectric response of the hyaluronate solutions. Dielectric changes at room temperatures were observed in aqueous solutions of hyaluronic acid as a function of concentration ranging from 0 to 350 mg/ml. The data indicated the existence of ordered phases in hyaluronate solutions at selective concentrations; that is, the hyaluronate molecule exhibited lyotropic type transitions. Hyaluronate solutions at 1.5 and 3 mg/ml concentrations were studies at various pH in the range 6-8 and at constant ionic strength of 0.1 units. The pH-dependent dielectric changes were observed in hyaluronate solutions at both tehse concentrations. A temperature-dependent transition in hyaluronate solution of 120 mg/ml concentration was observed at physiological

temperature. It was shown that this temperature dependent behavior could be related to the orientational polarizability term in the Debye theory of polar molecules in liquids.

The results of a study of the dielectric behavior of hyaluronate solutions were published in Biopolymers (Jani, Dahiya, and Roberts, 1980). The observed dielectric changes in hyaluronate solutions were shown to be related to the conformational changes in hyaluronic acid molecules. The peaks in the real and imaginary parts of the complex permittivity of hyaluronate solutions at 6 mg/ml and 120 mg/ml correspond to structured phases. The concentration dependence of the dielectric response in these solutions indicated that hyaluronic acid existed as a network in solution. This was in agreement with previous findings (Barrett and Harrington, 1977). It has been observed from the studies of linear depolarization ratio and the reversal coefficient that potassium hyaluronate in solution becomes an unpolarized uniformly refracting solvated sphere at approximately pH 8.0+ and ionic strength 0.1 (Barrett, 1979). The dielectric changes that were reported in the hyaluronate solutions (Jani et al., 1980) as a function of system pH were probably due to structural change, and thus, were similar in origin, and in agreement with, the linear depolarization ratio measurements.

It is important to notice that the hyaluronic acid molecule, in an environment other than physiological, may behave quite differently, and the observed pH-dependent transition may occur at different pH values (Balazs et al., 1977). The pH-dependent changes in dielectric response of hyaluronate solutions at concentrations of 1.5 and 3.0 mg/ml could be attributed to the corresponding changes in the internal viscosities of the solutions, a very important consideration in understanding the lubricating properties of hyaluronic acid in the joints of the human body.

To confirm that the observed pH-dependent dielectric changes in the hyaluronate solutions were due to viscosity changes in the solutions and not due to change in hydrogen ion concentration, the experiments were performed on the phosphate buffer solutions of different pH values without hyaluronic acid. The dielectric response of these solutions was linear with respect to pH values. This indicated that the curves obtained for the dielectric response of hyaluronate solutions as a function of system pH were characteristic of the hyaluronic acid, or at least characterized by the presence of the hyaluronic acid in the buffer solutions.

The dielectric studies of the hyaluronate solutions as a function of the pH and concentration, as mentioned above, have demonstrated that this behavior manifests itself in the frequency range near 9 GHz as a characteristic change in the dielectric response of the hyaluronic acid molecule. It is very important to study the dielectric response of hyaluronic acid molecules from human umbilical cord and the vitreous humor as a function of frequency and temperature. The microwave spectrometer appears to be the right tool for dielectric relaxation studies. The principal investigator and the co-principal investigator have established an experimental and theoretical approach to study the properties of the hyaluronic acid molecule.

RESEARCH DESIGN AND METHODS

DIELECTRIC STUDIES – THEORETICAL BACKGROUND

Relaxation may be defined as the time lag in the response of a system to a change in the physical forces to which it is subjected. The term relaxation mainly applies to linear systems where a response and a stimulus are proportional to one another in equilibrium. In dielectrics the stimulus is almost always an electrical field and the response is a polarization of the dielectric molecules. The time lag between the application of the field and the polarization of the molecules implies an irreversible degradation of free energy into heat. The relaxation time may be defined as the time in which this polarization is reduced to 1/e of its original value, where e is the natural logarithmic base.

The relaxation phenomenon was explained in the theory of polar molecultes (P. Debye, 1929) and it was shown that the polarization of a dielectric medium in an electric field might arise from the partial orientation of permanent molecular dipoles by the field as well as from the distortion of electronic orbits in the molecules. When a dielectric is subjected to an alternating field the orientation of the dipoles, and hence the polarization, will tend to reverse every time the polarity of the field changes. As long as the field remains low (<10 Hz), the polarization is able to follow the alternations of the field without any significant lag and the permittivity is independent of the frequency and has the same magnitude as in a static field. When the frequency is increased a point is reached beyond which the dipoles will no longer be able to rotate sufficiently rapidly so that their oscillations will begin to lag behind those of the field. As the frequency is further increased, the permanent dipoles will be completely unable to follow the field and the contribution to the static permittivity ( ) from this molecular process, the orientational polarization ceases and this usually occurs in the rf range (10 -10 Hz).

For frequencies in the infrared (10 -10 Hz) range, there is no contribution to from atomic or ionic polarization and the only polarization that contributes to is the electronic polarization. Therefore, the permittivity of a dielectric material decreases with increasing frequency. This process is known as dielectric dispersion.

At very high frequencies, the displacement vector *D*, electric field vector *E* and the dielectric constant of relationship *D* = *E* become complex quantities given by Where And

The quantities and are real and imaginary parts of the dielectric constant is the phase difference between *D* and *E*.

From Eq. (1), Comparing Eqs. (4) and (5)Therefore, Equation (8) represents the dielectric loss at a certain frequency.

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EXPERIMENTAL-APPARATUS

A microwave spectrometer in the x-band of frequencies has been designed in the physics department of Southeast Missouri State University. The block diagram of such a spectrometer is shown in Fig. 1. The spectrometer consists of a 2K25 klystron that is slightly tunable in the frequency range around 9.0 GHz. The klystron is powered by a HP715A power supply. The microwave signal produced by the klystron is guided by a waveguide to a directional coupler that divides this signal into two parts. A part of this signal reaches the microwave resonant cavity via an attenuator and a wavemeter. The attenuator is used to adjust the right amount of microwave power for the sample being studies. The function of the wavemeter is to determine the approximate frequency of the klystron by tuning it to the resonant frequency of the klystron. The other signal goes to a marker-mixer multiplier where it is mixed with harmonies of a standard frequency generator for precise frequency measurement. The next important device in this operation is a crystal detector which is used to detect the radio frequency wave. The oscillator is modulated.

The purpose of modulating the microwave signal is as follows: The unmodulated signal from the klystron gives a very narrow frequency interval as shown in the microwave power vs. frequency curve in need figures 2a. With a signal of this type, it is not easy to measure the shifts in frequencies and the Q-changes of the signal. Rapidly, a sawtooth sweep derived from the time base of a dual channel oscilloscope sweeps the klystron over the range of frequencies desired while simultaneously a chopper signal of 3 1 KHz is impressed upon the klystron repeller electrode to produce an ac signal of that frequency at the detector. This procedure allows the signal to be a.c. amplified and the method of phase coherent recovery used to significantly increase the signal – noise ratio. The modulated signal is shown in Fig. 2b and Fig 2c. As shown in Fig. 3a and 3b, the power absorption by the cavity is modulated to give a differential display of the resonance profile in the form of a butterfly. Figure 3c and 3d show the modulated signal before the sample under investigation is introduced into the cavity and after the sample has been introduced, respectively. The modulated signal is amplified by a preamplifier and displayed on the oscilloscope. Figure 4 shows a scan of the signal taken from the oscilloscope along with the frequency markers.

A cylindrical cavity operating in the TE mode has been designed. As the sample under investigation is introduced into the cavity it perturbs the electric field of the cavity and because of this there is a shift in the resonant frequency and the Q of the cavity changes. The frequency shifts and Q- changes of the signal are related to the real and imaginary parts of the dielectric constant through the Slater's perturbation equations as follows: (Dahiya et al, 1981)

AndWhere *E* is the field of the unperturbed cavity, *E* is the microwave field as applied to the cavity and E is the field of the sample itself, and v and V are the volumes of the sample and cavity, respectively.

The signal Q-change is further related to the width of the signal by (See meterfield et al referencies Where f is the resonant frequency of the system and W is the frequency separation of the half power peakes in Hertz, of the resonance half-power points.

The real and imaginary parts of the dielectric constant and are further related to the relaxation time ( ) using Debye's equations as follows: (P. Debye, 1929)The sample under investigation is put into a fine capillary tube. This tube is then inserted into the microwave resonant cavity along the symmetry axis of the cavity to ensure a sizeable perturbation of the microwave signal. A thermocouple is inserted into the capillary tube to make contact with the sample inside the tube to obtain the temperatures. The initial readings of the temperature and microwave frequency in the plates in the derivative profile are recorded.

The microwave resonant cavity is placed in a thermal bath. Temperature control of the sample is achieved by flushing nitrogen gas through a copper coil which is wrapped around the cavity, with the nitrogen gas passing through a heat exchanger. The sample under study is cooled or warmed to its desired temperature and this temperature is kept constant for some time to ensure thermal equilibrium between the sample and the cavity. The temperature is then allowed to vary by changing the flow of nitrogen gas around the cavity and that is taken in terms of the frequency shifts and width changes for each temperature change.

Those dates are streamed into a computer using lab view software. The frequency shifts and the width changes are recorded by using the frequency maker whose position is controlled manually by using the signal generator. Figure ( ) shows a complete set of frequency shifts and the width changes in MHz, with the markers center, and right peaks of the signal. These values of the frequency shifts and the width change as a function of temperature are used in Slater's equations and the values of the real and imaginary parts of the dielectric constant calculated at each temperature.

The microwave spectrometer is computerized to obtain the frequency shifts and width changes at desired temperature and to stream these data into an excel format wherein they are analyzed. This will add to the accuracy of measuring the dielectric relaxation mechanism in a sample of hyaluronic acid.

COMPUTATIONAL STUDIES – THEORECTICAL BACKGROUND (RELATING MICROSCOPTIC PROPERTIES TO MACROSCOPTIC MEASUREMENTS)

When a measurement of a physical property of a substance is made, the value obtained is a statistical average of the properties of an equilibrium distribution of the states of the substance.

Mathematically, this statement can be represented in the following manner: assume that F is the property whose value is sought. The value obtained will be an average where the integral formula indicates a continuous variable range and the summation formula indicates a discrete variable. P(x) is the probability of finding the system in state x. In a statistical thermodynamical calculation, P(x) is given by the Boltzmann distribution. In most physical and chemical problems, the probability distribution is unknown and needs to be determined by calculation.

The probability distribution is generated as a Markov chain of states. The relative number of states of each type will be commensurate with the Boltzmann distribution in the limit of very many trials, if certain conditions are met. The Markov chain can then be used to calculate average properties by summing over discrete states:

Here it is a counter used to identify and count states in the Markov chain.

The Markov chain itself is a sequence of states characterized by a single step probability matrix (q) where 0 for the change I (t) -> j (t + 1) and where (q) is independent of t, the counter of links in the Markov chain. It is also necessary that for all i. This statement means that the system isin some state and that all possible states are considered.

The convergence of the distribution of states in the Markov chain to the Boltzmann distribution when the Markov chain get very long is called the property of stochastic convergence. Stochastic convergence is not automatically obtained. Two conditions must be met for the Markov chain to be convergent.

1. It must be possible, and practical, for one to obtain any state j from an initial state I (ergodicity condition).
2. The probabilities P and q must be related so that
3. The matrix of probabilities (q) used in the generation of the Markov chain must be related to the equilibrium probability distribution in such a manner that the equilibrium probability distribution is preserved when the matrix multiplication in eq. [15] is performed (steady-state condition).

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The ergodicity and steady-state conditions are existence conditions for obtaining stochastic convergence. They are not prescriptive for determining a practical form of the elements of the matrix (q).

The Metropolis algorithm (Metropolis et al. 1953) is a practical way of prescribing a matrix (q) in order to generate a sequence of links in the Markov chain. The method is called "importance sampling."

The values of the probability matrix (q) are given by By defining the q's in terms of the equilibrium-state probabilities, the ergodicity and steady-state conditions are satisfied in principle. In order to practically implement the choice of q's described by eq. [17], the following procedure is used:

Starting from the current state, a new state is generated by randomly changing one of the variables describing the state (for example, a dihedral angle or an intermolecular distance). The probability of changing the state of the system from the old to the new is calculated by first finding the difference in energy between the two states. If the difference in energy is negative the new state is accepted and becomes the next link in the Markov chain. If the difference in energy is positive, the new state is less probable than the old state. In order to decide whether the new state should be accepted, a random number between 0 and 1 is generated. If this random number is less than or equal to the Boltzmann factor between the two states, the new state is accepted and becomes the next link in the Markov chain. Otherwise, the new state is rejected, and the next link in the Markov chain is repetition of the old state.

The Markov chain created by the Metropolis algorithm consists of a set of states with integer weights. Many physical and chemical applications can employ the Metropolis algorithm profitable. The classical Metropolis algorithm can be used if it is possible to modify the search ranges of the variables characterizing the states so that a probability of accepting a new state is approximately 50%. For example, in the modeling of liquid or gaseous state systems it is possible to either cut back or expand the maximum displacement of a molecule until the probability of accepting a new conformation achieves the desired level.

Not all problems are amenable to investigate by the classical Metropolis algorithm. Problems requiring an alternative approach frequently exhibit very complicated potential energy surfaces which have high barriers between regions in which thermodynamically acceptable conformations may be found. The problem associated with the application of the Metropolis algorithm is a practical problem of achieving ergodicity in searching for all likely states. Cutting back on the maximum search displacements for a set of variables may achieve the 50% probability for accepting new states at the cost of making many of the physically realistic states computationally inaccessible because the intermediate conformations connecting the regions of likely states are

themselves of extremely low probability. Typical problems of the class just described include the theoretical calculation of the distribution of conformations of polymers.

The application of the Metropolis algorithm to polymer systems will result in a low probability of accepting new conformations. The low probability of accepting new conformations will cause "trapping" of the calculation in some conformations. These conformations will have statistical weights unrepresentative of their values in a convergent calculation. Comparison of calculated properties based on these weights with experimentally determined properties will be meaningless.

In order to achieve a more vigorous development of the Markov chain by accepting more conformations, the umbrella sampling method (Valleau and Whittington 1977) can be used. The umbrella sampling method was originally developed to handle problems like free energy function evaluation and phase transitions – problems requiring the proper handling of large changes in the numbers involved. The conformational statistics of polymers also involves quantities such as conformational energies with widely varying values due to the non-bonded interaction among the functional groups in the molecule.

The Metropolis method is the basis of umbrella sampling. The Boltzmann factors for each state j is modified thus:

Where Pj' is the "Boltzmann" factor for state j after weighting. w is the weighting factor for state j, and Z and Z' are the partition functions for the unweighted and weighted states, respectively.

The previous equation can be rewritten to give

Where <1/w>' is the sum of the reciprocals of the weighting factors taken over the weighted state space. Since a sequence of weighted states is produced by umbrella sampling, it is necessary to relate the Markov chain of weighted states to a Markov chain of unweighted original states.

Any property is a thermodynamic average of state properties weighted by the probability of each state. Rewriting eq. [13], this statement can be expressed as:

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click here to view equation

Substitution for P gives

Install Equation Editor and double-
Click here to view equation.

In the calculation the reciprocal of the weighting factor for each weighted state is summed in order to normalize the calculation. The state properties over the weighted space are also tallied.

The value of P ' in the calculation is the integer weight of the conformation in the weighted state space. When the markov chain element is saved, the ratio P '/w is saved.

Umbrella sampling can be seen to take place by revising the state probabilities so that the states appear to be closer in energy than they really are. Since the decision whether to accept a new state is based on the modified probability, inclusion of the reciprocal of the weighting factor in the Markov chain will undo the changes introduced by the weighting factor so that the final probabilities as used will accurately reflect the state probabilities based on an unmodified Boltzmann distribution.

The states retained in the Markov chain generated by umbrella sampling will no longer have integer weights. Many of the state weights may be quite small reflecting their true physical probability. Their inclusion, however is necessary in order to provide a bridge between regions in state space in which there is a non-negligible probability of finding states. If the classical Metropolis algorithm were used instead, the very low probabilities of these bridging states would make it extremely unlikely that they could ever appear in the Markov chain and hence result in a region of state space being effectively inaccessible computationally.

COMPUTATIONAL METHOD

In order to calculate the energy of each conformation, the ECEPP empirical energy method will be used (Momany et at. 1974, 1975). Four contributions to the energy are identified – electrostatic, Lennard-Jones, hydrogen bond, and torsional. The electrostatic, Lennard-Jones, and hydrogen bond contributions are calculated between atom pairs. The electrostatic intereaction is calculated by means of Coulomb's law with CNDO (complete neglect of differential overlap) net charges at each of the atoms. Depending on the atomic types, one calculates either a Lennard-Jones nonbonded interaction using a 6-12 potential function OR a hydrogen bond interaction using a 10-12 potential function. Torsional energy contribution is added for every dihedral angle that is varied in the calculation.

Since the Boltzmann distributions will depend on the energies of the conformations, the sensitivity of the distributions and the properties calculated from them on the parameterization used to calculate the conformational energies will be studies. A frequently used alternative conformational energy method has been developed (Hopfinger 1973). Although it includes the same energetic as the Momany-Scheraga method, (momany et al 1974) the parameters differ, primarily in permitting closer contact between the atoms in the molecule. The potential availability of more geometries may have an effect on the Boltzmann distribution and the properties if the additional geometries have significant statistical weights. Furthermore, there may be changes in the relative energies of conformations found to be acceptable in either parametrization. Different relative energies will have a direct effect on the Boltzmann distribution.

The HSEA energy parameterization is an alternative empirical energy method that uses Kitaigorodsky 6-exp functions (Lemieux et al 1980). It ignores electrostatic and hydrogen bonding interactions and employs special torsional functions to handle the torsional potential energy at the anomeric linkage in the sugar chain.

Although the dihedral angels defining a polymer's conformation can take on all values from 0 through 360 degrees, not all of them should result in physically realistic conformations. In order to refine the selection of states, not all values will be available to each angel. Molecular fragments will be constructed in order to represent the local potential energy associated with each dihedral angle. The local energy calculations will cover the entire 360 degree turn of the dihedral angle. Only those angles corresponding to states of low energy will be used in the umbrella sampling calculation. The use of a restricted set of angle values taken in order to avoid looking at unreasonable conformations. Any conformation with a high local energy (due to steric hindrance) would remain at high energy and unlikely, even if possible, favorable long range interactions if the full molecule were incorporated.

Two types of preliminary calculations will be performed depending on the type of angle for which local energies are needed. If the angle is of large spatial extent – for instance an amide angle – it will be sufficient to calculate local energies around a single bond at either end of the amide as separate calculations. Low energy regions found in this way will be used to identify search ranges in the Monte Carlo calculation. Other dihedral angles, such as those in the 1-2, 1-3, or 1-4 linkages between carbohydrate rings, consist of a pair of angles whose values are closely coupled. In order to provide the best set of choices for the constituent angles, two angle probability density maps will be calculated, and inspection of these maps will be calculated, and inspection of these maps will disclose the best choices.

A simple weighting function can be used used in the umbrella sampling calculation:

Base was set to 4 and Low is a threshold value to be determined by numerical experiments on the molecule. The numerical experiment will seek a global minimum energy – or one close to it. This energy will be used to determine the threshold value. The effect of applying the weighting factors to the Boltzmann factors for the states will be to compress the energy range so that the ratio P '/P 'used to calculate a q 'would be of sufficient magnitude to permit a reasonable probability of accepting the new conformation.

The Markov chain of umbrella weighted conformations will be generated in a multi-start procedures. The Markov chain will be a composite of Markov chains from separate funs on the same polymer. Each run will generate 12,000 trials. The first 2000 trials will be discarded in order to allow the system to relax to a state that appears to be uncorrelated with the initial state. The remaining 10,000 conformations will be retained. The total number of conformations and the number discarded and retained are appropriate for 6 degrees of freedom. A total of 10 runs will

produce 100,000 conformations retained from property determination. For 6 degrees of freedom, the 100,000 conformations will allow nearly 17,000 trials per degree of freedom. The number of trials per degree of freedom compares favorably to the number of trials per degree of freedom in a problem in which the properties of a liquid of a liquid are modeled.

Three general classes of calculations will be performed in order to provide theoretical results that can be related to the dielectric constant and viscosity experiments in this work.

On all of the hyaluronic acid fragments, "gas phase" calculations will be performed. Following the procedure described previously, the molecular fragments will be subject to Monte Carlo calculations in which the only interactions assumed to be intramolecular. These calculations will provide the following preliminary information – a likely set of conformations and their statistical weights in the absence of the solvent environment and the likelihood of significant conformational change as a function of temperature since the computational temperature is a parameter that can be changed in the data set. The "gas phase" results will serve as reference points when salvation is included in the calculation. The solvent-free results will serve to indicate the extent of the effects induced by salvation.

"Gas phase" calculations can be performed in both field-free and in the presence of a static electric field. A static electric field can be included in the calculation in order to represent the orientational effects on the solute when a field is imposed in the dielectric constant experiments. Three additional degrees of freedom must be added into the calculation when a static field is imposed in order to represent the rotation of the molecule as a whole with respect to the field direction. The rotation of the molecule as a whole can be represented as continuum of angles since there should be no potential barriers. The orientational effects in the static field can be used to interpret relaxation experiments even though the dielectric properties experiments use an oscillating field. One of the components of the dielectric constant can be interpreted to relate directly to the properties of the system in a static field.

The next level of complexity in the calculations will be used to represent the solvation of the hyaluronic acid fragment. The solvent will be represented as a continuous jelly-like substance having the same dielectric constant as the real solvent in which the solute resides in a cavity. The cavity will have the same general geometry as the solute, but its walls will be at the van der Waals radii from the atomic centers in the solute. The cavity will appear to be a set of partial spheres in coalescence somewhat as a set of bubbles. Since the solute consists of functional groups of considerable polarity, charges will be induced on the walls of the cavity. The charges, in turn, will interact electrostatically with the polar groups in the solute. An additional contribution to the conformational energy will be incorporated into the calculation, and it may well affect the overall Boltzmann distribution of conformations. The extent of the effect of solvation in the cavity

representation will be discernable from comparision of the results obtained in this manner against those obtained in the "gas phase" calculation.

Both field-free and static field calculations will be performed in the cavity model in order to investigate the effect of solvation in the two different environments. Likewise, different computational temperatures will be used in order to investigate the sensitivity of conformation on temperature with the inclusion of solvation.

The third class of computation will include explicity solute molecules as discrete species. It is not uncommon to use from 5 to 200 solvent molecules with a solute fragment. If the number of degrees of freedom per solvent molecule is set to be 6 (for three translational degrees of freedom and three rotational degrees of freedom and non for vibrations), then it is necessary to account for 300 to 1200 degrees of freedom for the solvent alone. The solute fragment itself will have very many fewer – 12 to 25 or so including the rotation of the hydroxyl groups on the sugar rings which should be included in the calculation. In order to select the most significant solvent degrees of freedom – those associated with molecules that are the closest to the solute – a weighted selection procedure will be used (Owicki and Scheraga 1977). The closer the solvent molecule may be to the solute, the more likely it will be that a degree of freedom associated with that solvent molecule will be selected for change. Modelling the solvent's behavior will be straighter forward than modeling the solute since a degree of freedom for the solvent will be essentially continuous and the maximum change at any time can be easily modified in order to obtain the usual 50% acceptance rate for new conformations.

The degrees of freedom selected for the solute species will differ from those used in either the "gas phase" or cavity solvation model calculations. The three rotations of the solute as a whole will be included, regardless of whether one performs field free or static field calculations. The effect of the extended solvent structure in interaction with the solute can be discerned from the behavior of the solute as it rotates in its solvent cage. This behavior is directly related to relaxation measurements. The rotation of the solute is continuously variable and the maximum change can be adjusted to achieve 50% acceptance of new conformations. The rotation of the solute's ring hydroxyl groups will also be included since they have low barriers and their orientation has direct bearing on the overall structure of the solvent shell around the solute. The other dihedral angles in the molecules, such as those joining rings, will not be allowed to vary as part of the computational degrees of freedom of the solute. Since these angles are quite restricted to certain values, the change of an internal angle to a new value will be certain to cause innumerable steric collisions, since the new conformation of the solute will be imposed on the solvent shell which had relaxed to accommodate a different solute conformation. The internal degrees of freedom will be used as computational parameters that will be set prior to embarking on a series of Monte Carlo computations. The resulting Markov chain will be matched according

to the energies of the lowest energy conformation in each set and the relative weights of the chains adjusted accordingly in order to obtain a proper composite Boltzmann distribution.

1. Human Subjects
   Not applicable

2. Vertebrate Animals
   Not applicable

3. Consultants/Collaborators
   Letters and biographical sketches for co-principal investigator and consultant are attached. For biographical sketches please see pp. 9-12 and the letters are given in the Appendix.

4. Consortium/Contractual Arrangements
   Not applicable

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