DEGRADATION OF DNA BY INTENSE, NONCAVITATING ULTRASOUND

BY

STEPHEN ARTHUR HAWLEY
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY STEPHEN ARTHUR HAWLEY

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BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

Floyd Dunn

In Charge of Thesis

Head of Department

Recommendation concurred in†

Committee on Final Examination†

† Required for doctor's degree but not for master's.

D517

Table of Contents

I

Introduction

The research material presented in this dissertation is part of a growing effort in which studies in the biological and physical sciences are being allied to supplement knowledge in both areas. Empirical observation alone no longer serves to develop elemental ideas in biology, as the level of understanding at the present time focusses within the microcosmos that lies beyond the range of the light microscope.[12] It is now evident that new modes of study are necessary. The rapid advances in the fields of physics, electronics, and chemistry are responsible for the development of techniques capable of resolving patterns of function that are manifest on the molecular level. A major difficulty sometimes arises, however, in the application of concepts developed in connection with physical systems of a relatively homogeneous and simple nature to the complexity of the biological manifold. The utilization of ultrasound, a mechanical vibrational phenomenon above 15,000 cps, is not recent, but the techniques of generation and detection of these acoustic waves have only been a by-product of the progress in electronics that has occurred since the termination of World War II[8]. In these years, one of the most important applications of ultrasound has been its use as a surgical tool in the treatment of certain neurological disorders[4]. It was found that noncavitating focussed ultrasound could be employed to interact selectively with subcortical structures within the mammalian brain. The mechanism of interaction is not clearly understood, however, and it would be desirable to know more exactly how the

acoustic energy alters the neural tissue. Average values of the acoustic parameters have been determined in vivo[6]. However, due to the extreme complexity of neural tissue, these data do not elicit ideas relative to interactions on a molecular level. It seems likely that only by studying the interaction of sound on more elemental tissue components will a more profound understanding of the physical mechanism be derived. This dissertation describes one such study.

II

Discussion of the Problem

It has been known for some time that properly controlled, intense, non-cavitating ultrasound can produce changes in biological tissue[6]. For example, ultrasound in the frequency range near 1 Mc can be focussed and used in order to modify sites within the mammalian central nervous system[5]. Utilization of a focussed acoustic field to alter neural tissue possesses a major advantage over other methods, inasmuch as interior sites may be selectively altered without exposing intervening regions to damaging radiation[5, 6]. Acoustic fields may be applied in this manner to many types of biological structures provided that proper coupling may be made between the source and the desired points of irradiation. A major problem that remains for clarification in connection with the interaction of sound with biological components is the elucidation of the physical mechanism(s) that may be operative. It is known that a change which can be measured functionally appears as soon after administration of the appropriate ultrasonic dosage as a selected test can be made[2]. Also, it has been observed that the lesions, resulting from exposure to ultrasonic energy, appear histologically after a time delay of the order of 10 minutes. Thus, it appears reasonable to conclude that the primary action of ultrasound on neural tissue is at a structure too small to be seen in the light microscope, i. e., perhaps a macromolecule, and that the histologically observed lesion is the result normal physiological events following the disturbance. The possibility that the level of interaction in question is at the

macromolecular level does not seen unlikely, as it is well known that the integrity of biological organization is a manifestation of its macromolecular constituents. Therefore, it is desirable to study the relation of ultrasound to large molecules and the effects upon them, as a step toward a more clear understanding of the nature of interactions within higher levels of organization. Deoxyribosenucleic acid (DNA) was chosen as a suitable material for an initial study as it provides a well characterized system with respect to many physical parameters and is readily obtainable in sufficient quantities for experimentation.

To understand how a suspension of DNA might behave in a sonic field, it is necessary to know some of the physical-chemical aspects of this molecular species. The currently accepted structure of DNA in its physiological state is that of a long, double standed helix; each strand being a distinct polynucleic acid[14]. Hydrogen bonding is thought to provide the primary force responsible for the formation and stability of the helix. Each molecule behaves hydrodynamically as a long rigid rod with a diameter of 20 A, and a length of up to 65μ, the length being determined by the nature of the source and the procedure used in isolation[10]. Two modes of molecular alteration result when appropriate thermodynamic stresses are applied. The helical configuration may be reversibly destroyed, yielding two polynucleotides for each helix, by (1) increasing the solution pH; (2) increasing the solution temperature; or, (3) changing the ionic strength of the solvent. Each polynucleotide will then assume a random coil-like configuration, which may be restored to the helical form by carefully restoring the initial conditions to the solution. The helical structure may also be destabilized by adding compounds, such as formaldehyde, which are attracted to the electronegative bases and thereby

compete with the hydrogen bridging that holds the structure together. If the pH and temperature are changed drastically, the molecule may be irreversibly broken along the helix backbone due to the hydrolysis of chemical bonding. Molecular degradation may also be effected with ionizing radiation, cavitating sonic fields or by the production of shearing stresses along the molecule which are developed when a solution is forced through a fine capillary.

There are a number of ways in which a solution of DNA molecules might respond to the passage of ultrasound. If energy is properly coupled to the helical structure, it is feasible that partial strand separation will occur. This is a resonance phenomenon, and therefore frequency dependent, so that the possibility that strand separation will occur at any arbitrary frequency is small. However, the addition of formaldehyde would tend to preserve any strand separation that might occur in the response to action of the periodic field by preventing the reformation of hydrogen bonding. The kinetics of this change of state can easily be followed by observing the optical density of the solution. The absorbance of 260 mμ light by DNA is strongly dependent upon the physical state of the molecule. In the transition from helix to random coil, the optical density increases approximately 37%. This effect holds true for DNA of any degree of polymerization down to about seven nucleotide base pairs. Thus, if only a very small portion of the hydrogen bonded complex is ruptured, it should be revealed through the optical absorbance properties of DNA solutions containing formaldehyde[4].

Relative motion between solute and solvent resulting from mechanical stresses are probable since DNA in its native form has a density of about 1.7 times that of water. Any viscous interaction would result in strain distributions along

the molecular backbone and, if sufficiently great, would cause molecular degradation. If this effect is present, molecular degradation should be dependent upon the existing sound field intensity[7]. Examination of the molecular weight, prior to and following acoustic irradiation, would reveal the presence of molecular weight decreases.

Determination of the molecular weight can be readily accomplished by nature of the sedimentation properties of DNA in a high intensity centrifugal field. The sedimenting DNA forms a boundary with the non-sedimenting solvent and can be followed photographically as a function of time. The velocity of the boundary is directly related to the molecular weight and boundary sharpness is an indicator of the solution homogeneity[13].

Heat denaturation and sonic cavitation, which also affect molecular configuration[4], were avoided in this study in order to make the results as clear-cut as possible.

III

Experimental

A Apparatus

The experimental work is divided primarily into two parts: preparation and irradiation of the DNA samples; and molecular weight analysis of the irradiated material.

The irradiation apparatus is of a complex nature, incorporating several unique features. Sound is generated, focussed, detected, and absorbed within a single device. Irradiation of the DNA samples is carried out at 0.981 Mc with intense non-cavitating ultrasound. The mechanical vibration is generated by an X-cut quartz crystal driven at its resonant frequency (0.981 Mc) by an rf electronic driver (see Fig. 1). A spherical cut, planoconcave, polystyrene lens is coupled to the quartz plate by a thin layer of castor oil, and focusses the sound to the center of a cylindrical chamber. The acoustic energy that diverges beyond the focal volume is ultimately absorbed in castor oil which fills a large chamber terminating the acoustic transmission path. The castor oil in the final absorption chamber is separated from the rest of the sound path by a ρc rubber membrane[8]. Degassed distilled water is used to transmit and to couple the sound to the various elements of the path. Ports on either side of this chamber allow transmission of ultraviolet light in a direction normal to that of the sonic path and through the region of the focal volume.

Since it is desirable to irradiate small volumes at uniform acoustic intensities, a small cell is employed to support the samples (see Fig. 2). The cell, rectangular in shape, contains two chambers; one serves to hold the material to be irradiated, and the other (directly behind it relative to the direction of sound propagation) serves as a detector of the incident acoustic energy. Thin polyethylene membranes are used to form the front and rearmost walls as well as the interchamber partition of the sample-detector cell. The remaining walls of the sample chamber are composed of thin sheets of quartz, permitting the passage of ultraviolet light through the sample. A small constantan-copper thermocouple embedded in castor oil forms the acoustic detector within the chamber. This cell complex is fitted into the larger chamber, and is attached to a three dimensional coordinate system enabling it to be displaced within the acoustic field. By using the thermocouple as an acoustic detector, the sample cell may be positioned such that the focal volume produced by the lens falls within the DNA solution.

The radio frequency driver possesses negative feedback circuitry enabling the output to be stabilized at a predetermined voltage. A vacuum tube voltmeter, used to monitor the rf signal, is connected through a voltage divider in the output stage of the driver. Another feature of this system is the presence of a galvanometer, indicating the amount of feedback current necessary to produce stabilization of the output rf voltage and thereby allowing changes in electrical input impedance to be measured.

Ultraviolet light is generated by a xenon-mercury arc, and separated out by a monochromator to produce light with a wave length of 260 mμ. A 1P28 photomultiplier tube amplifies the emergent light energy so that it may be displayed on

a continous recording, trace storage oscilloscope. The absence of a comparator stage in the light detection circuit does not allow direct optical density readings and permits only relative values of absorbance amplitude to be observed.

Because the thermocouple generates a small transient direct current output, a breaker amplifier is necessary to amplify the detector voltage. This signal is then displayed on a continous recording oscilloscope[6]. The transient temperature change detected by the thermocouple embedded in the oil possesses two distinct phases. The first phase, which reaches an equilibrium value rapidly (in about 0.1 second), results from the conversion of acoustic energy into heat by the viscous forces acting between the wire and the immediately surrounding oil. The second phase exhibits an almost linear characteristic (for a pulse duration of approximately 1 second) and results from acoustic energy converted into heat by absorption in the medium surrounding the thermocouple junction. If the thermocouple wires are sufficiently small in diameter relative to the acoustic wavelength, the initial time rate of change of temperature from the second phase is related to the acoustic intensity by the relation

$$I = \frac{\rho c}{\mu} \left(\frac{dT}{dt}\right)_o$$

where ρc is the heat capacity per unit volume of the oil, I is the acoustic intensity, and μ is the acoustic intensity absorption coefficient per unit path length. This condition is satisfied in the experimental arrangement since the butt-welded constantan-copper thermocouple is fabricated from wires 0.003 inches in diameter and the acoustic wavelength in the oil at the operating frequency is approximately 1.5 mm[2,7].

The DNA samples were obtained from calf thymus and Bacillus subtilis.

The calf thymus DNA was obtained as a commercial preparation, and the Bacillus subtilis material was prepared according to a method described in 1961[11]. All nucleic acid stock is suspended in a saline citrate buffer (0.15M NaCl and 0.15M $Na_6H_5O_6$).

A model E Spinco ultracentrifuge fitted with ultraviolet optics is used for all molecular weight analyses and runs are made utilizing a sample cell containing the Kel-F centerpiece.

B Procedure

Stock solutions of the calf thymus and the Bacillus subtilis nucleic acids were prepared at 250μg/ml and diluted to appropriate concentrations prior to irradiation. The concentrations used in irradiation are fixed at 20μg/ml and 40μg/ml for the Bacillus subtilis and calf thymus DNA, respectively. These concentrations are used so that no further dilutions are necessary and subsequent dilution errors are not introduced into the ultracentifugal analyses. In addition, many of the physical parameters, which must be used in subsequent computations, for the sedimentation analyses of DNA have been obtained in this concentration range. Other advantages of using low concentration of the nucleic acids are the reduction of the possibility of intermolecular interaction and the conservation of sample material.

Calf thymus DNA is more heterogeneous with regard to molecular weight distribution than the Bacillus subtilis DNA. Since the effectiveness of the sedimentation analyses depends upon the contrast between the boundary and the solvent, slightly higher concentrations of the calf thymus DNA are advantageous.

The cell is positioned for sonic irradiation at a predetermined crystal

voltage using the thermocouple as an acoustic detector. The photometric observation system is then positioned by eye at some visible wavelength and then adjusted at 260 mμ with the guidance of the photomultiplier response.

The sample cell is cleaned and 1.2 ml of solution is carefully pipetted into it. An irradiation time is selected and the thermocouple response and optical density are monitored while the sound is on. The sample is removed, following irradiation, and carefully stored in the cold until molecular weight determinations can be made.

During each irradiation series, a control sample is prepared, receiving identical treatment as other samples except that it is never exposed to acoustic energy.

A few samples of calf thymus DNA were prepared and irradiated in increasing formaldehyde gradients, but as no sonically induced optical density changes were present (even at concentrations just below that which would result in spontaneous denaturation) the use of the optical density monitoring system was discontinued[4].

Measurement of the temperature changes within the region of the sample are made by using a sensitive mercury thermometer as well as the calibrated thermoelectric detector.

Acoustic intensity measurements are made by moving the thermocouple into the region of the focal volume, and recording the time rate of temperature change[6]. (See section III)

The standard procedure for sedimentation velocity molecular weight determination is used. The rotor speed is 36,500 rpm with ultraviolet light exposures made every four minutes for twenty minute runs.

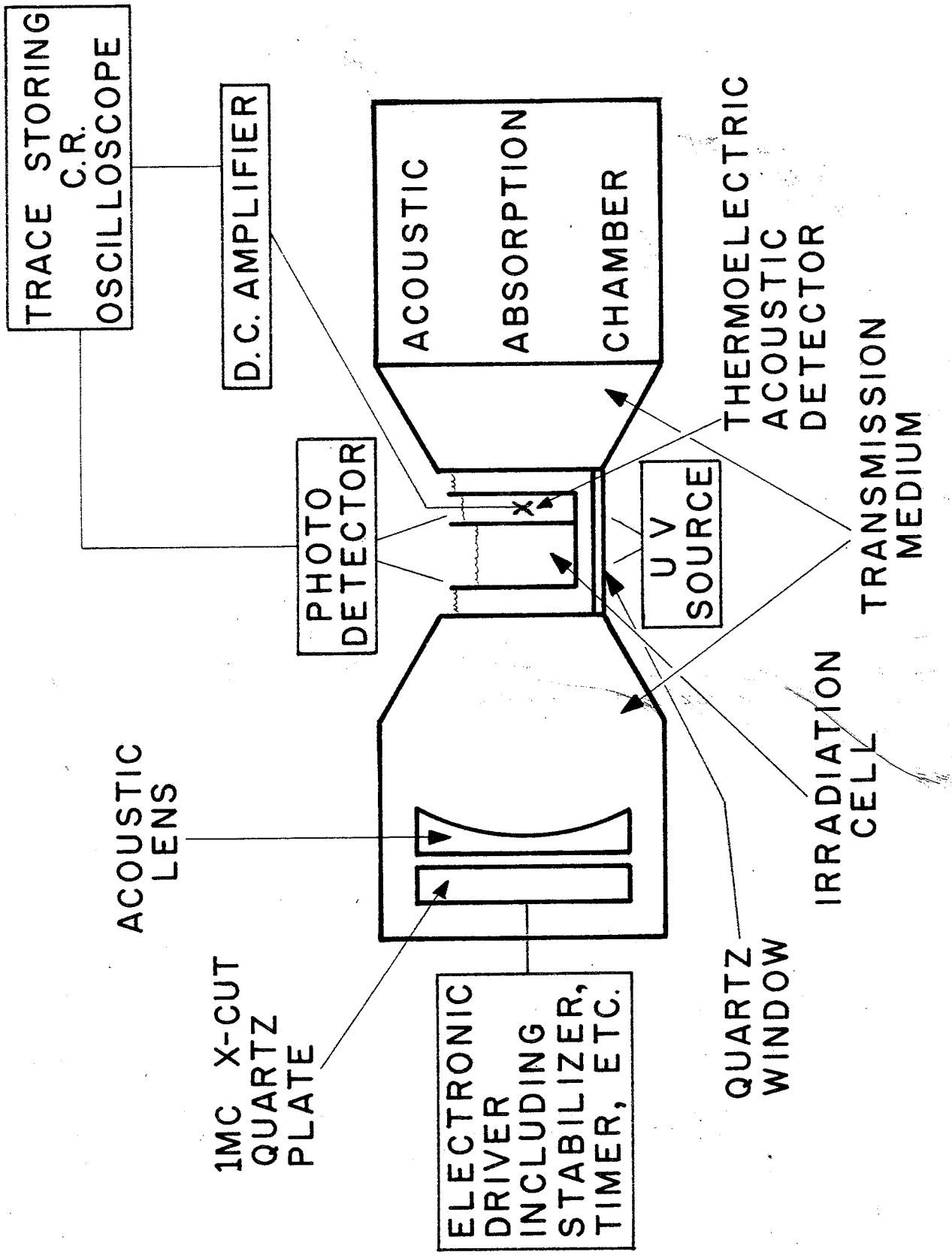

Fig. 1. Schematic illustration of experimental arrangement.

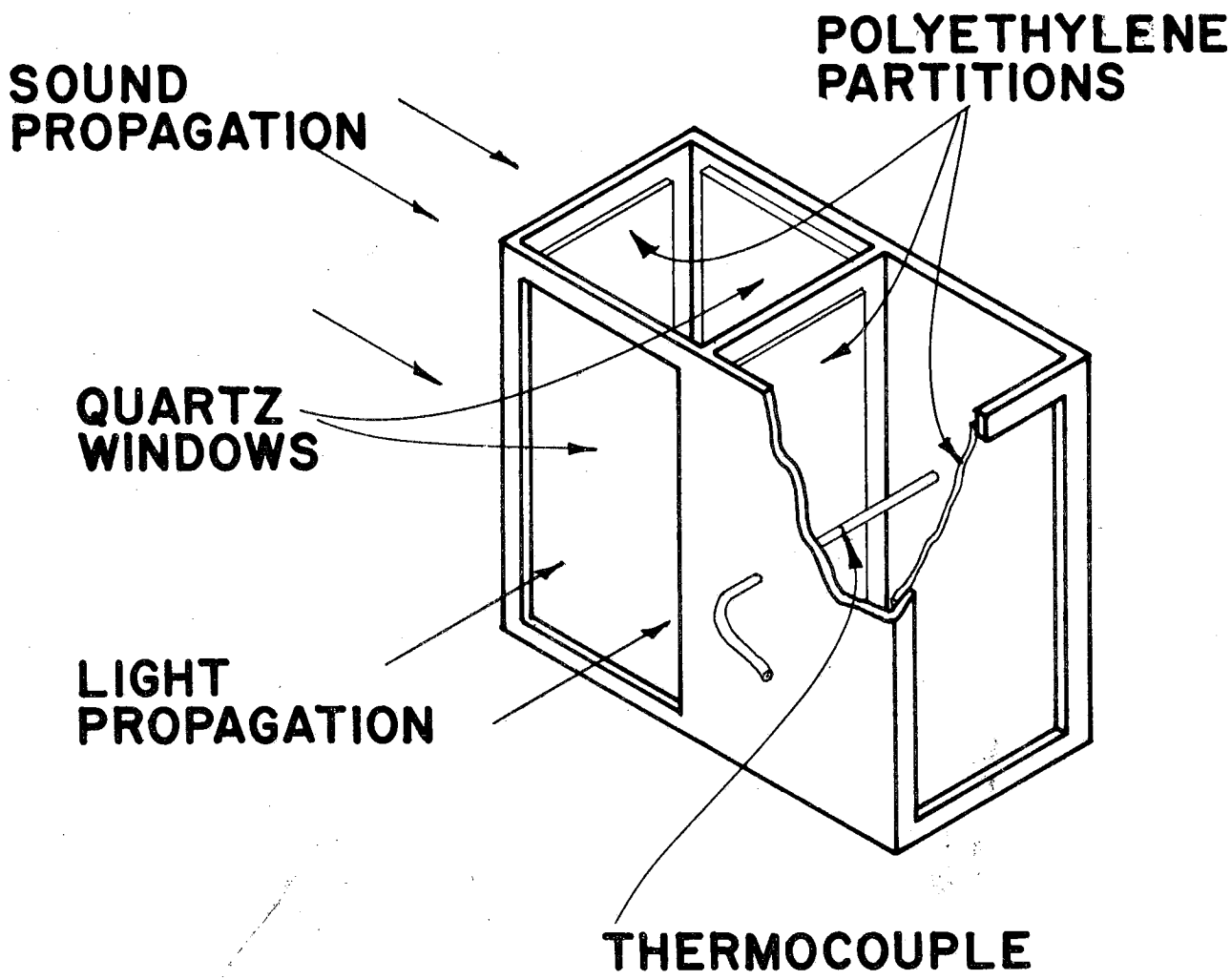

Fig. 2. Sample cell, cutaway to illustrate position of thermocouple.

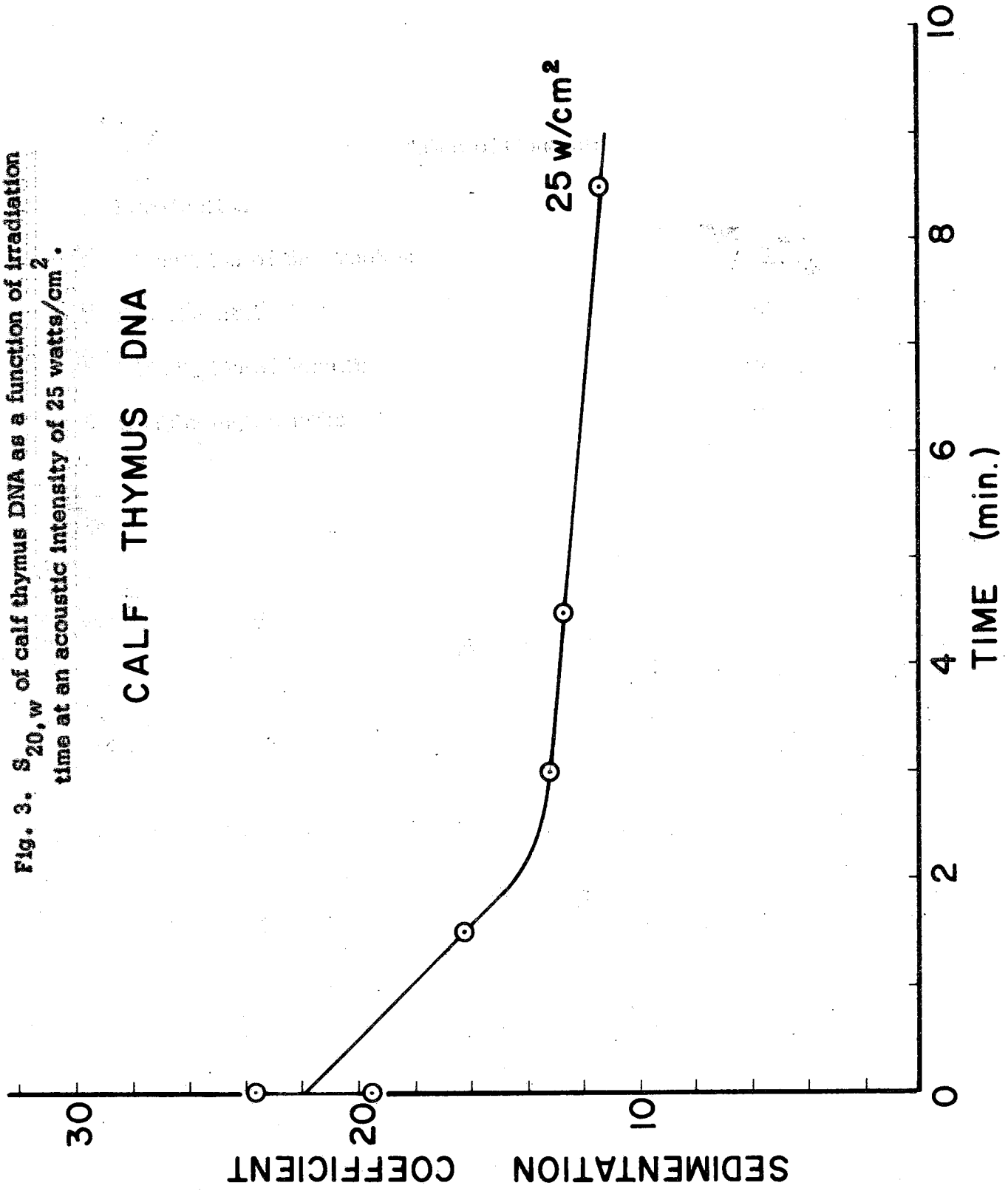

Fig. 3. $S_{20,w}$ of calf thymus DNA as a function of irradiation time at an acoustic intensity of 25 watts/cm^2.

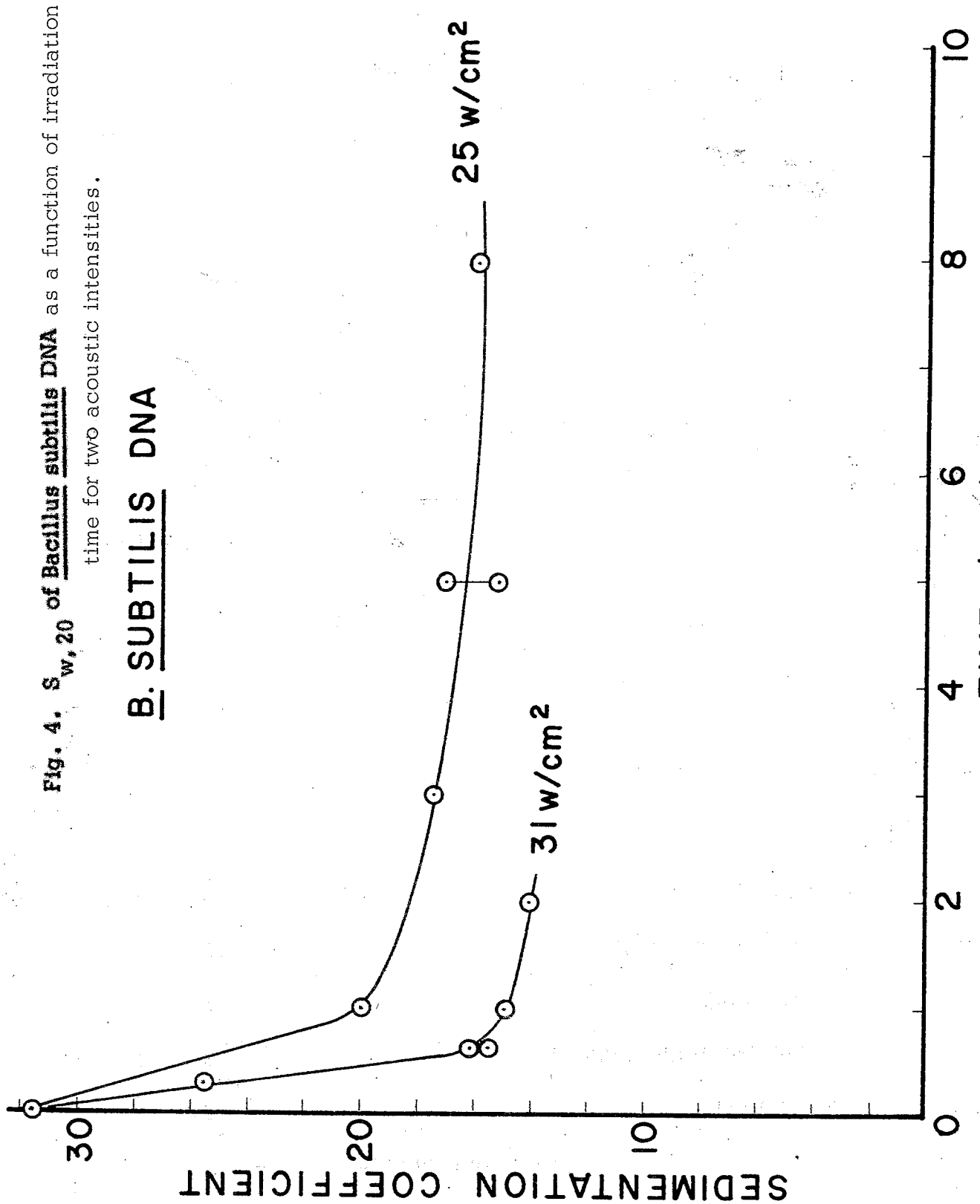

Fig. 4. $S_{w,20}$ of *Bacillus subtilis* DNA as a function of irradiation time for two acoustic intensities.

TABLE I - Sedimentation analysis data

SAMPLE MATERIAL	ACOUSTIC INTENSITY	IRRADIATION TIME	$S_{20,w}$	$M \times 10^{-6}$
B. subtilis DNA	25 watts/cm^2	1.0 min	19.8	5.44
"	"	3.0 min	17.5	3.92
"	"	5.0 min	15.0 →17.2	3.83
"	"	7 55/60 min	16.0	3.12
"	"	NATIVE	31.6	20.9
B. subtilis DNA	31 watts/cm^2	19 sec	25.4	10.8
"	"	39 sec	15.2	3.24
"	"	1.0 min	14.9	2.58
"	"	2.0 min	14.1	2.22
"	"	NATIVE	31.6	20.9
calf thymus DNA	25 watts/cm^2	1.5 min	16.3	3.23
"	"	3.0 min	12.4 →13.0	1.74
"	"	4.5 min	13.7 →12.5	1.56
"	"	8.5 min	11.2	1.19
"	"	NATIVE	19.3	5.12

IV

Discussion of Results

The experimental results presented in Table I and that in the graphs of Figures 3 and 4 represent molecular weight analyses of samples from several series of irradiations.

The sedimentation coefficient $S_{20,w}$ (velocity per unit field of force relative to water) is related to the molecular weight, and for DNA this relation[1] is

$$S_{20,w} = .063M^{.37}$$

Further, based on the Watson-Crick model for DNA, the molecular weight is proportional to the molecular length. If an average value of 350 molecular weight units per nucleotide base pair is assumed, the value for molecular weight units per Angstrom lengths is about 250.

In the curves of Figures 3 and 4, the ordinate is the sedimentation coefficient in Svedbergs (1 Svedberg = 10^{-13} sec) and the abscissa is irradiation time in minutes. Since molecular weight determines the sedimentation constant, these curves reveal the presence of molecular degradation in the sound field. However, it is difficult to determine the nature of the physical interaction from these data. Random attack, such as might result from the formation of free radicals, is ruled out by the kinetics of degradation. It can be seen that the amount of cleavage approaches zero after approximately one minute of irradiation.

The rapid initial decrease followed by a subsequent leveling off may be interpreted in the following way. For each value of acoustic intensity, there

exists a value of molecular length below which no degradation occurs. That is, DNA molecules longer than a particular length are vulnerable to the action of the acoustic energy of a specified intensity and those of lesser length remain unaffected. Assuming breakage is the result of a strain distribution along the molecule produced by shearing stresses along its length, the degree of degradation should follow a non random process. This is analogous to the experimentally observed results obtained by flowing DNA solutions through small capillaries[9]. The midpoint along the molecule is the position of most probable cleavage. Thus, if a monodisperse solution of DNA is irradiated at an appropriate acoustic intensity, all the molecules will be sheared until none of vulnerable length remain in the solution. Since the initial rapid decrease is seen for both calf thymus and Bacillus subtilis DNA, it may be concluded that molecular length is sufficient in both cases to produce cleavage for the levels of acoustic intensity employed. If degradation does proceed by the above mechanism, it would be anticipated that the asymptotic leveling off of molecular weight as a function of time to be independent of the DNA source. This can be seen to be approximately true by comparing the data obtained for calf thymus and Bacillus subtilis DNA at 25 watts/cm^2. However, since calf thymus DNA is a more heterogeneous preparation all molecules would not be equally vulnerable to cleavage at the chosen intensity and the final average molecular weight would be somewhat lower than Bacillus subtilis material under the same exposure conditions.

Since prior to this work it was generally believed[15] that molecular degradation was not possible in the absence of cavitation, the experimental procedure was arranged to avoid cavitation fields. All solutions were carefully degassed in order that the threshold of cavitation be well beyond the working acoustic

intensities[6]. In addition, several methods were available to detect the presence of cavitation. For example, the response of the thermoelectric detector is altered drastically at the onset of cavitation, due to the diminishing amount of energy passing through the cell to the detector. Another mode of detection lies in the fact that cavitation bubbles increase the optical density to 260mμ light. Since both optical and thermoelectric responses were monitored during irradiation and no cavitation was indicated, it is safe to assume that all results were obtained in a noncavitating acoustic field.

V

Concluding Remarks

The important finding of the work described is the demonstration that interactions of noncavitating ultrasound within the molecular domain are possible. This helps to substantiate the hypothesis that the physical mechanism(s) of intense, noncavitating acoustic interaction in tissue structures are manifested, at least in part, at submicroscopic sites. However, experimental determination of many of the interesting physical parameters have not yet been completed as only preliminary aspects of the study have been considered. It seems likely that through further exploration, not only will firmer bases of the understanding of interactions in tissue components be established, but also development of a useful tool with which macromolecules may be studied will be initiated.

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