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BY

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THESIS

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This investigation was prompted by the need for an explanation for the functional and structural changes observed when living tissues are irradiated with intense noncavitating ultrasound. Previous research into the nature of these interactions suggested that the ultrasound may act at the molecular level and thus the study of the interactions of intense noncavitating ultrasound with solutions of biomacromolecules was undertaken as an elementary approach to the more complex study of the effects in tissue. Solutions of five enzymes (α-chymotrypsin, trypsin, lactate dehydrogenase, aldolase, and ribonuclease) were irradiated with noncavitating ultrasound under a variety of physical and chemical conditions. Frequencies were in the range 1 to 30 MHz, intensities ranged from 0.5 to 1000 w/cm^2 depending on the frequency, and irradiation times varied from 0.1 to 600 seconds depending on the intensity. The temperature, pH, and concentration of the solutions depended on the properties of the enzyme being studied and, where appropriate, these variables were altered to determine if they affected the results of the irradiation. Two types of ultrasonic irradiations were performed on the enzyme solutions. In the first type of irradiation the solutions were exposed to the noncavitating ultrasound and then analyzed to determine if any permanent change in the enzyme molecules was produced. Analytical procedures employed included measurements of the enzymatic activity, the u.v. absorption spectrum, the specific optical rotation, the sedimentation coefficient, and the use of thin-layer chromatography. In the second type of irradiation the enzyme is irradiated in a solution of

its substrate, while it is catalyzing a specific biochemical reaction. This technique makes possible the detection of any reversible changes produced by interaction with noncavitating ultrasound, for by simultaneously irradiating and monitoring the rate of the biochemical reaction it is possible to observe the occurrence of changes in the enzyme molecule which affect its catalytic activity. A specially constructed irradiation system was built into a spectrophotometer so that the irradiated enzyme reactions could be monitored spectrophotometrically.

The results of both types of irradiations, which employed noncavitating ultrasound at dose levels sufficient to cause extensive structural and functional damage in tissue, show that the noncavitating ultrasound has no effect on either the structure or the function of the enzymes. Trypsin, α-chymotrypsin, and lactate dehydrogenase were also exposed to cavitating ultrasound and these exposures resulted in loss of enzymatic activity and structural damage to the enzyme molecules. Thus it is concluded that cavitation is a necessary condition if damage is to be produced by ultrasound in enzyme solutions *in vitro*. Previous reports of inactivation of enzyme solutions by noncavitating ultrasound are shown to be the result of an artifact of the experimental procedure and not due to ultrasound.

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I. INTRODUCTION

Ultrasonic energy transmitted by a liquid medium has been employed in a variety of ways in biological and medical research, but understanding of the processes by which biological structures are altered upon interaction with ultrasound is limited. The intent of this study is to contribute and interpret new facts with the hope that understanding of these interactions will be improved.

To facilitate discussion of what has been discovered and what remains enigmatic, it is useful to divide the interactions of biological structures with ultrasound into two categories: those in which cavitation occurs and those in which cavitation is absent. Some discussion of the nature of ultrasound and cavitation will aid in making the distinction between these two categories.

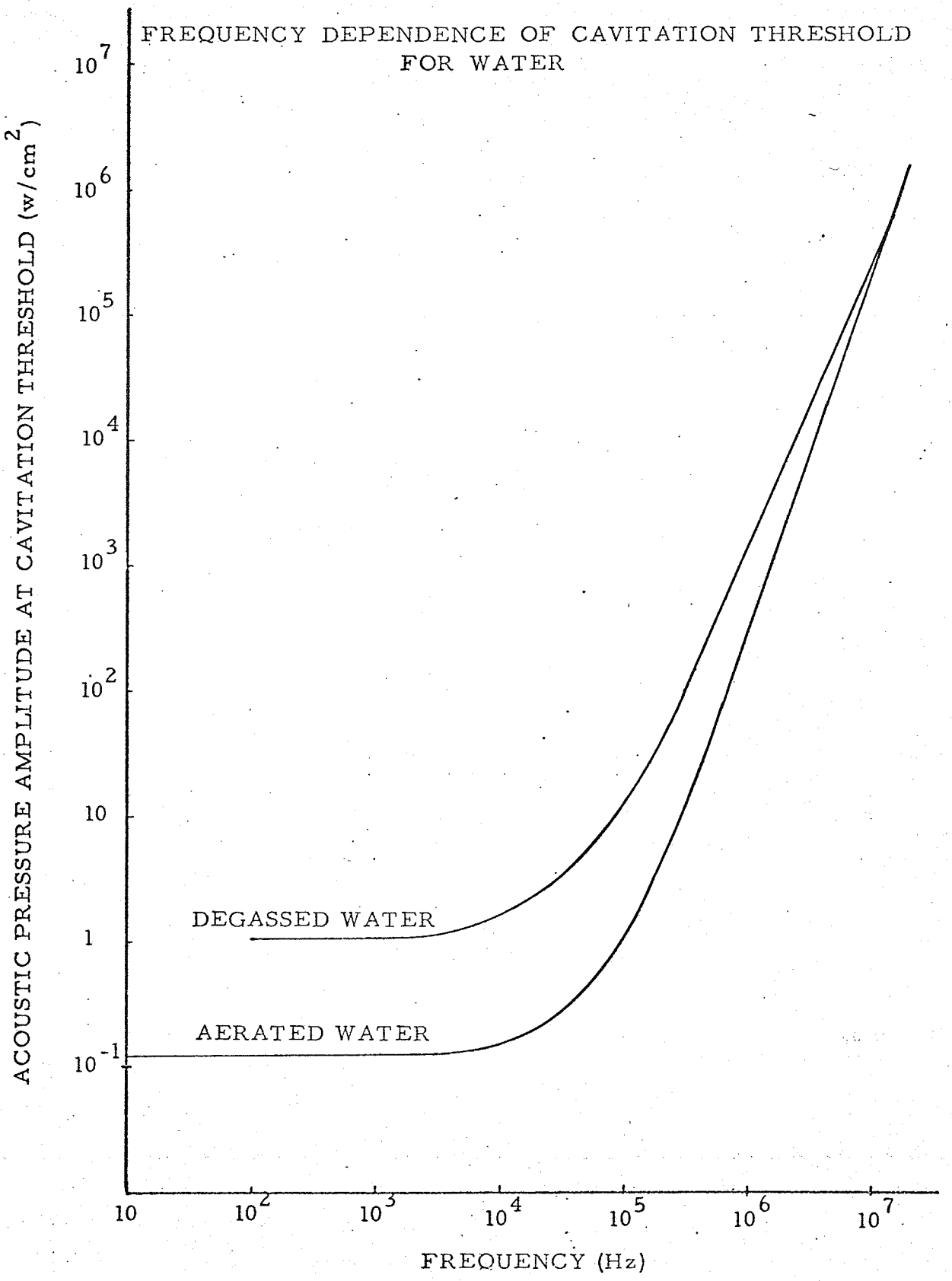
Ultrasound is usually defined to be sound at frequencies beyond the upper limit of the human hearing range, that is, greater than 20 kHz. The upper frequency limit of the useful ultrasonic range in liquid media is about 2 kMHz so that the entire range available for study extends five orders of magnitude. Like all acoustic waves, ultrasonic waves result from vibration and are manifest as an alternating series of compressions and rarefactions of the media surrounding the vibrating element. Among the several types of transducers used to produce ultrasound, the most common for frequencies greater than 100 kHz is the piezoelectric vibrator (Kinsler and Frey, 1962). When piezoelectric crystals (such as X-cut quartz) are excited electrically, they transduce the electrical energy into mechanical deformations which are then propagated through the surrounding medium as an acoustic wave with the same frequency as the excitation voltage. Little mechanical output is

achieved unless the crystal is excited near the odd harmonics of its fundamental resonant frequency. Thus, a crystal with a 1 MHz fundamental resonant frequency delivers a maximum acoustic output when driven at 1 MHz and is also capable of producing significant levels of ultrasound when driven at 3 MHz, 5 MHz, 7 MHz, 9 MHz, etc. The intensity of the ultrasound produced decreases as $1/n^2$ where n is an integer representing the harmonic of the fundamental resonant frequency. Ultrasound exhibits all the usual properties of wave phenomena, viz., it may be diffracted, reflected and refracted and the intensity of an ultrasonic wave is attenuated by acoustic absorption losses in the medium through which it is transmitted. Focussing ultrasound by acoustic lenses is an application of the refraction property which provides a method for increasing the acoustic intensity in a relatively small volume at some distance from the transducer without appreciable disturbance to the intervening medium.

Cavitation is the formation and collapse of cavities within a liquid medium which occurs when the hydrostatic pressure during the rarefaction phase of an ultrasonic wave falls below some threshold value. The cavitation threshold depends on properties of the liquid medium, such as its viscosity and density; properties of the environment of the medium, such as temperature, pressure and the amount of dissolved gas; and properties of the ultrasound, such as the frequency and intensity. Figure 1 (Fry and Dunn, 1962a) shows the variation of the acoustic intensity cavitation threshold with frequency for degassed and aerated water at room temperature. The threshold is increased by increasing hydrostatic pressure and decreased by increasing temperature (Blake, 1949). The cavitation threshold also may be decreased by the presence of particulate matter in the medium which provides weak spots

FIGURE 1

FREQUENCY DEPENDENCE OF CAVITATION THRESHOLD FOR WATER



in the liquid structure (so-called cavitation nuclei) where a bubble is more easily formed. Aqueous solutions of polymers whose viscosities are greater than water tend to inhibit bubble formation and, hence, have greater cavitation thresholds (Briggs et al., 1947). Although information on cavitation thresholds is not available for most aqueous solutions, use of Figure 1 and knowledge of the direction in which the other factors mentioned affect the cavitation threshold, allow the proper frequency and intensity to be selected to produce or prevent cavitation. Thus, in this work, when employing intense noncavitating ultrasound, the samples were degassed and the lowest frequency used was 1 MHz.

Two types of effects are responsible for the results observed when cavitating ultrasound interacts with biological structures. (1) When a cavitation bubble collapses, the resulting shock wave causes intense hydrodynamic shearing forces in the vicinity of the collapsed cavity. Familiar devices which apply this effect are the commercially available ultrasonic cleaning baths and the cavitators used in many microbiology and biochemistry laboratories to break open bacterial cells.

The second type of effect caused by cavitation is chemical in nature. (2) Oxidations, reductions, free radical formation, luminescence, polymerization and depolymerization are some of the chemical effects produced by cavitation in liquids and solutions and several explanations have been proposed for these phenomena. One explanation assumes that the liquid-vapor boundary within a forming cavity is in a highly reactive state and that chemical bonds are broken to yield ions and free radicals at the cavity surface (Marboe and Weyl, 1950). Another explanation postulates a separation of charge upon cavity formation and a subsequent discharge to produce ions in

the vapor-filled cavity (Lindström, 1955). The "Hot Spot" explanation (Fitzgerald et al., 1956) holds that the chemical reactions take place in the gas phase inside a vibrating cavity ("resonance bubble") where the theory predicts that temperatures higher than 1000°C can be expected. Other investigators believe that the high temperatures and pressures resulting from cavity collapse are responsible for the chemical effects observed (Weissler and Cooper, 1948). All the proposed explanations account in some way for the observed phenomena but, as yet, no single theory is firmly established. Most investigators agree that the specific chemical reactions observed depend not only on the solvent and solute but also on the dissolved gases present.

Synthesis of the organic compounds formaldehyde and hydrocyanic acid was observed when water saturated with nitrogen, hydrogen and carbon monoxide was ultrasonically cavitated (El'piner, 1964a). Also, when aqueous solutions of acetic, succinic and glutaric acids were saturated with nitrogen and hydrogen and ultrasonically cavitated, the amino acids glycine, aspartic acid and glutamic acid, respectively, were formed (El'piner and Sokol'skaya, 1960). In other experiments, ultrasonically produced free radicals have been used to initiate the copolymerization of organic compounds such as methyl methacrylate and acrylonitrile (El'piner, 1964b). However, aside from these special reactions in which synthesis and polymerization occur, the interaction of biological structures with cavitating ultrasound produced degradation of the structure involved. In most cases, the degradation cause cannot be assigned exclusively either to the mechanical shearing effects or to chemical effects but it seems likely that the former play a greater role in breaking down large structures such as tissue fragments or bacterial cells in suspension and that the latter are largely responsible for changes in small molecules.

Since the principle part of this study does not deal with cavitating ultrasound, no further discussion of the numerous reports of degradation of macromolecules, microorganisms and plant and animal cells will be given here. El'piner (1964c) gives a comprehensive discussion of these interactions. Remarks on reported interactions of enzymes with cavitating ultrasound are deferred to Chapter III for purposes of comparison with the results of this study.

The second category of interactions between biological structures and ultrasound includes all the interactions occurring in the absence of cavitation. The research in this category can be subdivided, according to the intensity of the ultrasound employed, into applications of "low-level" and "high-level" ultrasound. The average acoustic intensities for "low-level" applications are approximately 10^{-3} w/cm^2 while "high-level" intensities may exceed 10^3 w/cm^2. Research applications of "high-level" or intense non-cavitating ultrasound are concerned with the changes which the acoustic energy produces in the biological structure under study while "low-level" research applications are concerned with deducing information about biological structures from the transmission properties of the ultrasound in the structure.

When an ultrasonic wave encounters an interface between two media of different characteristic acoustic impedances (the product of the sound velocity in, and density of, a medium), part of the acoustic energy is transmitted, part absorbed and part reflected. Since the acoustic impedances of various tissues differ, ultrasound incident on a structure composed of several tissues will be reflected from the tissue interfaces. Procedures have been developed to detect these reflections and convert them to a direct visual display. These techniques for ultrasonic tissue visualization

are used, in situations where X-rays would be useless, to determine the gross structure and movements of soft tissues (Fry and Dunn,1962a; Kelly, 1965).

The absorption of weak noncavitating ultrasound has important applications in biological research. For a plane progressive ultrasonic wave moving in the positive direction, the intensity I at any position X in the medium is given by

$$I = I_o e^{-2\alpha X} \qquad (1)$$

where I_o is the intensity at X = 0 and α is the acoustic amplitude absorption coefficient per unit path length. Absorption measurements (Fry and Dunn, 1962b) for a particular medium are usually reported in graphical form with α per wavelength plotted versus the ultrasonic frequency. Curves of this type for tissues or solutions of biomacromolecules are useful in the selection of intensities and irradiation times when studying other interactions of ultrasound with the same tissue or solution of biomacromolecules. An example of the use of absorption data for protein solutions is given later in this chapter.

Ultrasonic absorption measurements are also employed in relaxation spectrometry (Eigen and Hammes, 1963) where the ultrasonic wave serves as a periodic perturbation which loses part of its energy when the solution through which it is propagated is unable to return ("relax") to its pre-perturbation structure or chemical composition during the period of the disturbance. Since the period of the wave is the inverse of the frequency, if a relaxational response to the perturbation occurs, at some frequency the relaxation lag will cause a maximum attenuation (α per wavelength) of the ultrasonic wave. This frequency is called the relaxation frequency and its

reciprocal is the relaxation time. Theory relates the relaxation time to the rate constant and, thus, locating the frequency where a maximum α per wavelength occurs provides a method for determining rate constants for processes having half-times between 10^{-5} to 5×10^{-10} seconds. Examples of biochemical processes whose rates have been determined by ultrasonic relaxation spectrometry are the helix-to-random coil conformational change in polylysine (Hammes and Burke, 1965) and the urease catalyzed breakdown of urea into ammonia and carbon dioxide (Saksena, 1965). Saksena found that formation of the urease-urea complex produced a maximum in the α per wavelength versus frequency curve at 8 MHz and that the calculated rate constants agreed well with the results obtained by other methods.

Intense noncavitating ultrasound has been employed to produce changes in biological structures ranging in complexity from living tissues to simpler systems such as an individual species of biomacromolecule *in vitro*. Glandular tissues (Curtis, 1965; Krumins *et al.*, 1965), skeletal muscle (Welkowitz and Fry, 1956) and nervous tissue (Dunn, 1956; W. J. Fry, 1958) all have been structurally altered by intense noncavitating ultrasound and, in the case of nervous tissue, changes in physiological function have been observed as well. The most sophisticated and complete studies were those carried out on the mammalian central nervous system (W. J. Fry, 1958). With the selection of the proper combination of intensity and irradiation time, it is possible to produce reversible changes in physiological function without producing an observable lesion (F. J. Fry *et al.*, 1958); however, most of the neurological research has made use of permanent lesions. For example, recent research in quantitative neuroanatomy made use of ultrasonically produced lesions to selectively block certain nerve paths in the brain (W. J. Fry *et al.*,

1964). Intense ultrasound has also been used successfully in human neurosurgery to alleviate the symptoms of Parkinson's disease (W. J. Fry et al., 1958). The great advantage of intense ultrasound in neurosurgery is that through focussing, a very high acoustic intensity can be placed accurately, deep within the brain if necessary, to produce a small lesion without damaging the surrounding brain tissue.

Despite numerous reports of the effects of intense ultrasound in tissues, little is known about the mechanisms of interaction responsible for these effects. Cavitation can be eliminated as a possible explanation for the effects observed in nervous tissue not only because the acoustic intensity was not great enough to cavitate the tissue at the frequency used, but also because experiments performed under hydrostatic pressures high enough to prevent cavitation produced lesions similar to those produced in the absence of the applied pressure (W. J. Fry et al., 1951). Three types of experiments showed that the temperature increase which occurs in tissue during ultrasonic irradiation cannot be responsible for the damage observed. First, irradiations of tissue containing imbedded thermocouples showed that the temperature increase never reached a damaging level. Irradiations at low temperatures, where the highest temperature reached was considerably below the animal's normal body temperature, resulted in damage indistinguishable from body temperature experiments. Irradiations in which exposures were temporally spaced to allow cooling between exposures and which individually produced no damage had a cumulative effect equivalent to a single long exposure with an accompanying temperature increase (W. J. Fry et al., 1958). In addition, theoretical considerations eliminated the possibility of microscopic "hot spots" being developed at interfaces within the tissue (W. J. Fry et al.,

1951). A specific mechanical mechanism (Welkowitz, 1955), which proposed that unidirectional forces caused elastic failure of tissue structural components when these components are displaced from their equilibrium positions, has been shown to be untenable on the basis of more recent experimental evidence (Dunn, 1957).

Although physiological changes resulting from irradiations of the central nervous system (limb paralysis, for example) can be detected almost immediately after irradiation, histological examination of irradiated brain tissue from animals sacrificed from 5 to 10 min. after irradiation shows no evidence of lesions. Tissue taken from animals sacrificed at times longer than 10 min. after irradiation, however, shows progressively greater evidence of lesion formation (W. J. Fry, 1958). The absence of detectable structural changes in a functionally damaged tissue suggests that the primary site at which the ultrasound acts is a submicroscopic structure, perhaps a macromolecule. Histochemical studies, in which stains specific for certain enzyme catalyzed reactions were employed, show that in brain tissue severely damaged by ultrasonic irradiation, the enzyme systems were completely and irreversibly destroyed and that a considerable decrease in enzyme activity and a delayed resynthesis of enzymes occurred in tissue which was damaged but did not become necrotic (Bostelmann, 1962). This report of enzyme inactivation in brain tissue irradiated with noncavitating ultrasound reinforced the notion that the submicroscopic site affected may be a macromolecule although, admittedly, a variety of indirect effects could equally well explain the observed loss of enzyme activity. For example, Bostelmann used unfocussed ultrasound irradiated through the animal's skull and, since bone is a highly absorbing acoustic medium, it is possible that the enzyme inactivations result

from heat denaturation rather than the direct action of ultrasound.

Although degradation of organic polymers with noncavitating ultrasound had been reported earlier (Melville and Murray, 1950), the first direct evidence for damage to a biomacromolecule by noncavitating ultrasound were the reports of *in vitro* denaturation of solutions of trypsin (Stefanović *et al.*, 1958-59) and salivary diastase (Stefanović *et al.*, 1960) using 3 Mc ultrasound with intensities of 2.5 and 1.5 w/cm^2, respectively. The only other experiments in which biomacromolecules *in vitro* have been shown to interact with intense noncavitating ultrasound are the reports of DNA degradation (Frontali, 1962; Hawley *et al.*, 1963). Though few in number, these reports demonstrate that biomacromolecules *in vitro* can be directly damaged by noncavitating ultrasound. This does not mean that the action of ultrasound *in vivo* is necessarily directly on macromolecular structure. However, *in vitro* studies offer a simple and promising starting point in the search for a mechanism to study the more complex interactions occurring in tissue. Determination of the levels of structure at which ultrasound acts is one approach to the mechanism problem; another approach being the identification of the physical process(es) involved.

Just as in tissue studies, temperature increase upon irradiation and the chemical effects and shearing forces of cavitation can be eliminated as possible mechanisms. Because ultrasound consists of a rapidly fluctuating succession of compressions and rarefactions propagating through the medium, mechanisms which invoke hydrodynamic shearing forces possess an intuitive appeal. Indeed, hydrodynamic shearing forces have been shown to be responsible for DNA degradation when solutions of DNA were forced through capillaries (Levinthal and Davison, 1961) and it was on the basis of this report that a

mechanism was suggested proposing shearing forces due to relative motion between DNA molecules and solvent molecules as the cause of DNA degradation by intense noncavitating ultrasound (Hawley et al., 1963).

Before speculating further on possible mechanisms, it is appropriate to obtain a better knowledge of what is involved in the degradations observed. More information is required about the types of chemical bonds broken, about whether there is a limiting molecular size, i.e., a smallest size moelcule that can be degraded, and whether reversible changes occur in the presence of noncavitating ultrasound.

Five enzymes, α-chymotrypsin, trypsin, aldolase, lactate dehydrogenase and ribonuclease, were selected for study. The decision to study enzymes was prompted by several considerations. First, previous investigations (mentioned above) indicated that enzymes are susceptible to inactivation by noncavitating ultrasound (Stefanovic et al., 1958-59; Stefanovic et al., 1960; and Bostelmann, 1962). Secondly, enzymes are important biomacromolecules to study since their inactivation may be fatal for the tissue cells which depend on their normal functioning. Finally, it was felt that more information could be derived from a study of the interactions with these relatively well-characterized biomacromolecules than any other class of macromolecules. Enzymes have a complex globular structure and, thus, present elements of primary, secondary and tertiary protein structure to interact with ultrasound. Furthermore, their specific catalytic abilities are very sensitive to any alteration of structure which affects the active site and this provides a sensitive indicator for even very subtle changes in the native enzyme's conformation. In addition, denaturation and/or degradation is reliably reflected by changes in such physico-chemical properties of the enzymes as sedimentation

behavior, optical rotation and the ultraviolet absorption spectrum. Another advantage of using the particular enzymes selected is that they are available in purified form and, thus, a homogenous sample of uniform size, shape and molecular weight is studied rather than a distribution of these characteristics as is the case for most samples of other classes of macromolecules.

The five enzymes chosen for study, although basically globular proteins, exhibit a variety of enzymatic properties and diversities of structural detail. There is slightly more than a ten-fold range in molecular weight, from 13,683 for ribonuclease to 149,000 for aldolase. Two, α-chymotrypsin and trypsin, are proteolytic enzymes; aldolase and lactate dehydrogenase are glycolytic enzymes; and ribonuclease catalyzes the hydrolysis of ribonucleic acid (RNA). Ribonuclease is a single polypeptide chain of 124 amino acids, the sequence of which is completely determined (Hirs et al., 1960) and the eight half-cystines of the molecule form four disulfide bridges whose positions are also known (Sparkman et al., 1960). A three-dimensional electron density map at a resolution of 4 Å has been reported (Harker et al., 1964) but, as yet, a complete three-dimensional image of the polypeptide chain in crystalline ribonuclease is not available. The work of many investigators has strongly indicated the participation of the histidine residues at positions 12 and 119 in the active site (Mathias et al., 1964). The proteolytic enzymes, α-chymotrypsin and trypsin, also apparently have two histidine residues involved in their active sites. In these two enzymes, however, there is a serine residue involved as well. The complete sequences of the 242 amino acids of α-chymotrypsin (Keil and Šorm, 1964 and Hartly, 1964) and of the 223 amino acids of trypsin (Walsh et al., 1964) have been determined, but the three-dimensional structures are still unknown. The enterokinase

catalyzed activation of trypsinogen to trypsin involves the cleavage of a hexopeptide from the amino terminus of the single polypeptide chain of the zymogen and, thus, trypsin also consists of a single polypeptide chain. When chymotrypsinogen, on the other hand, is activated to α-chymotrypsin by trypsin, two dipeptides are cleaved from within the zymogen chain so that the result is three chains joined together by disulfide bridges (Desnuelle and Rovery, 1961). Aldolase and lactate dehydrogenase are also multichain enzymes having three and four subunits, respectively. Aldolase can be dissociated into three highly unfolded subunits of nearly the same size in acidic solutions, and reassociated into an enzymatically active form extremely similar to native aldolase by neutralizing the acidic solution of subunits (Deal et al., 1963; Stellwagen and Schachman, 1962).

Lactate dehydrogenase is a tetramer thought to be composed of two types of subunits in one of five different combinations, the particular combination of the subunit arrangement in a particular isozyme depending upon the physiological needs of the tissue from which it is isolated. Thus, one kind of subunit (designated "H") predominates in heart muscle, where pyruvate is oxidized aerobically via the Krebs cycle, and the second type of subunit (designated "M") predominates in skeletal muscle and tissues where metabolism is largely anaerobic and pyruvate is reduced to lactate. It is known that a sulfhydral group is involved in the active site and it is probable that there is one active site per subunit, i.e., four active sites per molecule (Fondy et al., 1965). Also, there is evidence that the reaction mechanism involves a ternary complex of enzyme, cofactor and substrate which is very sensitive to changes in enzyme conformation (Fromm, 1961; McKay and Kaplan, 1961).

The manner in which intense noncavitating ultrasound can be expected to affect the activity and structure of these five enzymes is difficult to

predict because previous investigations in vitro (Stefanović et al., 1958-59 and Stefanović et al., 1960) did not include detailed analyses of the inactivated enzymes. However, some limits can be placed on the possibilities for effects produced by ultrasound based on the physical conditions provided by the ultrasound employed and the behavior of enzyme solutions under the influence of similar physical conditions of different origin. For example, one might inquire whether the peak pressure developed in a compression region of an enzyme solution which is transmitting ultrasonic waves is sufficient to denature the enzyme. The inactivation of trypsin and α-chymotrypsin by exposure to high pressure has been investigated (Curl and Jansen, 1950) and it was found that below pH 4, pressures up to 9200 Kg/cm^2 did not affect the enzymes but that, e.g., at pH 5.0-5.2, 50 per cent inactivation of both enzymes was produced within 5 min. by a pressure of 7750 Kg/cm^2. Since the highest pressure produced in a compression region for the most intense ultrasound employed in the work of this report is about 55 Kg/cm^2, it is doubtful if the pressure developed can affect any of the enzyme solutions.

Calculation of the frictional force experienced by an enzyme molecule due to relative motion between the moelcule and the solvent demonstrates that this force is too small to break covalent bonds. The calculation is made in the following way. The motions of the particles of a liquid medium which transmits a plane ultrasonic wave are described by the peak values of the alternating components of particle displacement, D; velocity, V; and acceleration, A:

$$D = \frac{1}{\omega} \sqrt{2I/\rho_o c} \qquad (2)$$

$$V = \sqrt{2I/\rho_o c} \qquad (3)$$

$$A = \omega \sqrt{2I/\rho_o c} \tag{4}$$

where I is the intensity, ω is the angular frequency, ρ_o is the density of the medium and c is the sound velocity in the medium. Consider a mass M with density ρ and volume v, suspended in a liquid of density ρ_o. Assume that the frictional force exerted on M is $R(\dot{d} - \dot{X})$ where $\dot{d}$ is the particle velocity of the liquid, $\dot{X}$ is the particle velocity of M and R is a constant which depends upon the structure and orientation of M but which is independent of the ultrasonic frequency. For a one-dimensional treatment, the motion of M is described by the following differential equation (Fry, 1952):

$$\ddot{X} + \frac{R}{M_e}\dot{X} = \frac{R}{M_e}\dot{d} + \frac{m_e}{M_e}\ddot{d} \tag{5}$$

where M_e is the "effective" mass of M and m_e is the "effective" mass of an element of the suspending liquid of identical shape and size as M. For a rigid sphere of radius r, the "effective" mass is the sum of the mass M of the sphere and the mass m of the displaced liquid multiplied by a factor f, where (Lamb, 1932)

$$f = \frac{1}{2} + (9/4r)(2\ \eta_o/\omega\ \rho_o)^{1/2} \tag{6}$$

and where η_o is the coefficient of viscosity of the liquid and ρ_o is its density. Thus,

$$M_e = M + fm \tag{7}$$

$$m_e = m + fm \tag{8}$$

For sinusoidal perturbation,

$$\ddot{X} = j\ \omega\ \dot{X}\ , \quad \ddot{d} = j\ \omega\ \dot{d} \quad \text{and so}$$

$$j\,\omega\,\dot{X} + \frac{R}{M_e}\,\dot{X} = \frac{R}{M_e}\,\dot{d} + j\,\omega\,\frac{m_e}{M_e}\,\dot{d}$$

Thus,

$$\frac{\dot{X}}{\dot{d}} = \frac{\frac{R}{M_e} + j\,\omega\,\frac{m_e}{M_e}}{\frac{R}{M_e} + j\,\omega} = \frac{1 + j\,\omega\,\frac{m_e}{R}}{1 + j\,\omega\,\frac{M_e}{R}} \qquad (9)$$

As ω approaches 0,

$$\frac{\dot{X}}{\dot{d}} = 1 \quad \text{and there is no relative motion.}$$

As ω approaches ∞,

$$\frac{\dot{X}}{\dot{d}} = \frac{m_e}{M_e} \qquad (10)$$

and the relative motion depends upon the ratio of the effective masses. Substituting (6) into (7) and (8) and then substituting the resulting expressions into (10) and taking the limit as ω approaches ∞ gives

$$\lim_{\omega \to \infty} \frac{\dot{X}}{\dot{d}} = \lim_{\omega \to \infty} \frac{\rho_o v\left[\frac{3}{2} + (9/4r)(2\eta_o/\omega\rho_o)^{1/2}\right]}{v\left(e + \rho_o\left[\frac{1}{2} + (9/4r)(2\eta_o/\omega\rho_o)^{1/2}\right]\right.} = \frac{\frac{3}{2}\rho_o}{e + \frac{1}{2}\rho_o}$$

Thus,

$$\lim_{\omega \to \infty} \frac{\dot{X}}{\dot{d}} = \frac{3}{1 + 2\,e/\rho_o} \qquad (11)$$

For enzymes $e/\rho_o \sim 1.4$ and using this value in (11) gives

$$\frac{\dot{X}}{\dot{d}} = \frac{3}{3.8} \sim 0.8 \qquad (12)$$

Letting V_r represent the relative velocity,

$$V_r = \dot{d} - \dot{X}$$

which, for an enzyme molecule, from (12), gives

$$V_r = 0.2\ \dot{d} \tag{13}$$

for the relative velocity between enzyme molecules and their solvent. Evaluating $\dot{d} = V$ from (3) using the maximum intensity obtainable with equipment used in this work, $I = 1000\ w/cm^2$, and the values of $\rho_o = 1$ and $c = 1.46 \times 10^5$ cm/sec for water,

$$\dot{d} = V = 370 \text{ cm/sec}$$

and substituting this value in (13) gives

$$V_r = 74 \text{ cm/sec} \tag{14}$$

Calculation of the frictional force is made from a modified form of Stoke's equation for the frictional force on a sphere of radius R_o,

$$f = 6\pi\ \eta_o\ R_o\ V_r\ k \tag{15}$$

where η_o is the liquid viscosity and $k = f/f_o$ for the enzyme molecule. Using the value of V_r from (14), 10^{-2} poise for η_o, a value of 35 Å for R_o calculated for aldolase (the enzyme of highest molecular weight in this work) from its molecular weight and partial specific volume, and 1.3 for k for aldolase (Deal et al., 1963) (the largest value for any of the enzymes studied), Equation (15) yields a frictional force due to relative motion of $f = 6.9 \times 10^{-6}$ dynes. When this value is compared to the tensile force which a c-c bond can resist, 8.1×10^{-4} dynes (Levinthal and Davison, 1961), it is apparent that relative motion results in a frictional force too small to break c-c bonds or other covalent bonds of about the same strength.

Because interest in measurements of acoustic absorption coefficients of biomacromolecules is relatively new and because relatively large amounts of expensive material are required for the measurements, there is little acoustic absorption data available for protein solutions and there is none available for enzyme solutions. Therefore, the ultrasonic absorption of hemoglobin solutions (Carstensen and Schwan, 1959) and of bovine serum albumin solutions (Hawley, 1966) are used to estimate the ultrasonic absorption in enzyme solutions. Since hemoglobin and bovine serum albumin (BSA) are globular proteins and their molecular weights (65,000 and 67,000, respectively) are midway in the molecular weight range of the enzymes studied, it is felt that the estimate is a reasonable one. The α (in excess of the solvent) for a 1 per cent solution of BSA at 11 MHz is 2.42×10^{-2} cm^{-1}. Use of this value for α and an I_o of 1000 w/cm^2 in Equation (1) gives 50 watts per cm^3 for the power dissipated per cm^3. 50 $watts/cm^3$ is equivalent to 12 cal cm^{-3} sec^{-1}, and when this latter value is divided by the number of moles of enzyme per cm^3 in a 1 per cent solution, it gives the energy in cal $mole^{-1}$ sec^{-1} absorbed by the enzyme. Calculations for 1 per cent aldolase and 1 per cent ribonuclease yield energy absorptions of 1.8×10^5 K cal $mole^{-1}$ sec^{-1} and 1.6×10^4 K cal $mole^{-1}$ sec^{-1}, respectively. These values are enormous compared to the strengths of covalent bonds, 50 to 100 K cal/mole, or the activation free energy for protein denaturation, $\Delta F^{\ddagger} \sim 25$ K cal/mole (Joly, 1965). Similar calculations for a frequency of 1 MHz and an initial intensity of 75 w/cm^2 and calculations based on the absorption coefficient for hemoglobin (Hb) are given in Table 1.

Applying the same estimates to the enzymes with higher molecular weights results in correspondingly larger absorptions as shown by the calculation for 1 per cent aldolase in the text above. The conclusions inferred from these

TABLE 1

ESTIMATED ABSORPTION OF ULTRASONIC ENERGY RIBONUCLEASE

Frequency (MHz)	Intensity (w/cm^2)	α (Hb) (cm^{-1})	Energy Absorbed K cal $mole^{-1}$ sec^{-1}	α (BSA) (cm^{-1})	Energy Absorbed K cal $mole^{-1}$ sec^{-1}
1	75	5×10^{-4}	8.0	1×10^{-3}	16.0
11	10^3	1.43×10^{-2}	8.9×10^3	2.42×10^{-2}	1.6×10^4

calculations are that at 11 MHz and 10^3 w/cm^2, ample energy is absorbed to denature enzymes molecules and that, at 1 MHz and 75 w/cm^2, irradiations of several seconds will also supply sufficient energy for denaturation. Whether or not denaturation does occur depends on the mechanisms of energy absorption and energy dissipation. Neither of these mechanisms is understood.

Two types of studies were carried out on the five enzymes described above. The first type of study consisted of irradiating enzyme solutions with intense noncavitating ultrasound and analyzing by various techniques for changes in the biochemical and physical properties of the enzymes. The enzyme activity, ultraviolet absorption spectrum, optical rotation and sedimentation velocity behavior of enzyme solutions irradiated at the frequencies and intensities given in Table 1 were compared to the results of similar analyses of unirradiated control samples. Loss of enzyme activity indicates denaturation, but methods of analysis other than activity assays are required to determine whether the enzyme molecules have been extensively degraded or if comparatively minor alterations in the tertiary structure of the enzyme molecules are responsible for the loss of catalytic ability. Fragmentation of the enzyme molecules can be detected by sedimentation velocity analysis, and cleavage of oligopeptides or individual amino acids from the enzyme molecules can be detected by paper or thin-layer chromatography. Denaturation, which results in changes in the secondary and tertiary structure of the enzymes, may be detected by observing changes in the ultraviolet absorption spectrum, specific optical rotation and sedimentation coefficient. The phenolic and indole side chains of the amino acids tyrosine and tryptophan are principally responsible for the absorption maximum near 280 mμ in proteins. Changes in the native secondary and tertiary structure of an enzyme molecule,

which result in changes in the environment of these chromophores, may give rise to a shift in the wavelength of the absorption maximum and/or changes in the magnitude of the absorption at various wavelengths (Wetlaufer, 1962). Similarly, an unfolding of the enzyme molecule which results in a change in the number and orientation of the centers of assymetry responsible for the specific optical rotation of an enzyme solution will cause a change in the specific optical rotation (Urnes and Doty, 1961). Changes in the size and shape of an enzyme molecule can be detected by determining changes in the value of the sedimentation coefficient. For example, an unfolding of the molecules will result in an increased frictional force on the sedimenting molecules which is reflected as a decrease in the sedimentation coefficient. Thus, the combination of these analytical techniques permits the examination for a wide range of alterations in enzyme structure and function, from extensive degradation to subtle changes in enzyme conformation. The temperature, pH and ionic strength of the solutions in which the enzymes were irradiated were altered in some cases to determine the effects of these variables on the interaction between the enzyme molecules and the ultrasound.

The second set of experiments was designed to determine if reversible changes in enzyme structure occur under the influence of ultrasound, which are completed too rapidly for detection by the analytical techniques employed in the first experiments. A procedure which offers the possibility of detecting ultrasonically-induced reversible structural changes in enzyme molecules is to irradiate the enzyme while it is performing its catalytic function in a biochemical reaction. For, if the structural change upsets the comparatively weak bonding between enzyme and substrate at the active site, a change in the reaction rate is to be expected. If the active site

is distorted in a way that inhibits the formation of the enzyme-substrate complex, the reaction rate will decrease; however, if some fortuitous rearrangement occurs that results in a more active catalytic site, the reaction rate will increase. A special reaction chamber is required to study the kinetics of ultrasonically-irradiated reactions, one in which the reaction can be monitored and irradiated with ultrasound, simultaneously. Spectrophotometry is a convenient method for monitoring enzyme catalyzed reactions and is applicable to the reactions catalyzed by the five enzymes chosen for this study. Accordingly, a reaction cell arranged for ultrasonic irradiation of enzyme catalyzed reactions was built into a spectrophotometer.

Although no ultrasonic absorption measurements have been made on any of the substrates used in these reaction irradiation experiments, measurements on similar substances indicate that the absorption of ultrasound by the substrate molecules is negligible compared to the absorption of water (Barrett, 1966). The ultrasonic absorption in hemoglobin and BSA solutions is directly proportional to concentration for concentrations less than 10 per cent (Carstensen and Schwan, 1959; Hawley, 1966) and, thus, the absorption per enzyme molecule in dilute solutions is the same as that estimated for solutions of concentrations up to 10 per cent. Table 2 lists estimates of the energy absorbed per mole per second for ribonuclease for the frequencies and some representative intensities used in the irradiations of enzyme catalyzed reactions. The estimates are based on the acoustic amplitude absorption coefficients of BSA and were calculated by the same procedure used for Table 1. Values for energy absorbed per mole per second based on hemoglobin absorption coefficients are about half the values listed.

Recently, an absorption at 8 MHz has been reported for the system urease-urea-water (Saksena, 1965) that is more than four times greater than

TABLE 2

ESTIMATED ABSORPTION OF ULTRASONIC ENERGY BY RIBONUCLEASE

Frequency (MHz)	Intensity (w/cm^2)	α (BSA) (cm^{-1})	Energy Absorbed K cal $mole^{-1}$ sec^{-1}
1	5	1.0×10^{-3}	3.3
9	20	1.9×10^{-2}	245
27	1	8.0×10^{-2}	205

the sum of the individual absorption values at 8 MHz for urease-water and urea-water. Saksena attributes the increased absorption to the enzyme-substrate complex. If the interpretation given to the high absorption value is valid and the phenomenon is a general one, i.e., all enzyme-substrate complexes have greater absorption values in the frequency range 1 to 30 MHz than the enzyme in water, then the values of energy absorbed per mole per second for an enzyme catalyzing a reaction will be greater than estimates like the ones in Table 2. Hence, judging from the estimated energy absorbed by the enzymes, it appears likely that the irradiation of enzyme catalyzed reactions with intense noncavitating ultrasound will provide an interesting kinetic approach to the interpretation of the interaction of ultrasound with enzyme molecules in solution.

The two experimental approaches outlined above, detailed analysis of enzyme solutions which have been irradiated with intense noncavitating ultrasound and ultrasonic irradiation of enzyme catalyzed reactions, were followed by experiments using cavitating ultrasound. These cavitation experiments were performed to supplement the relatively few detailed reports given in the literature and for comparison to the results obtained with noncavitating ultrasound.

II. MATERIALS AND METHODS

A. Biochemical Materials

1. Reagents

All inorganic reagents were prepared from reagent grade chemicals using distilled deionized water. The reagents used, the companies that supplied them and, where pertinent, the grade are given in Table 3.

2. Enzymes and Enzyme Solutions

Alpha chymotrypsin, trypsin, aldolase and lactate dehydrogenase were obtained from the Worthington Biochemical Corporation. Ribonuclease was obtained from Sigma Chemical Company. The following descriptions of the preparative procedures employed by the suppliers show the quality of the enzymes used in the investigation.

Alpha chymotrypsin was three times crystallized from the activation product of three times crystallized chymotrypsinogen (Northrup et al., 1948a), dialyzed salt free and lyophilized. Trypsin was prepared according to the method described by Northrup, Kunitz and Herriott for trypsinogen (Northrup et al., 1948b). It was drystallized twice, dialyzed salt free and lyophilized. The aldolase was prepared from rabbit muscle according to the method of Taylor et al. (1948). It was twice crystallized and supplied as a suspension in 52 per cent saturated ammonium sulfate, pH 7.8. Small amounts of pyruvic kinase and lactate dehydrogenase were present in this preparation. Lactate dehydrogenase was prepared from rabbit muscle, crystallized twice by the method of Racker (1952) and supplied as a suspension in 50 per cent saturated ammonium sulfate. A small amount of pyruvic kinase was present in this preparation. Ribonuclease (bovine pancreatic) was crystallized by the McDonald modification (McDonald, 1948) of the Kunitz procedure (Kunitz, 1940). It is a three times crystallized, protease free, essentially salt free preparation roughly chromatographed to increase the amount of fraction A

TABLE 3

REAGENTS

REAGENT	SUPPLIER	GRADE
Potassium Chloride	Mallinckrodt Chemical Works	Analytical Reagent
Sodium Chloride	" " "	
Dibasic Sodium Phosphate	" " "	
Monobasic Potassium Phosphate	" " "	
Sodium Hydroxide	" " "	
Hydrochloric Acid ("Acculute concentrate for Standard Solutions)	Anachemia Chemicals Ltd.	Analytical Reagent
Tris (hydroxymethyl) amino methane ("Trisma" base)	Sigma Chemical Co.	Reagent
Hydrazine Sulfate	Eastman Kodak Co.	Reagent
Reduced Nicotinamide - Adenine dinucleotide, Sodium Salt	P-L Biochemicals, Inc.	
Disodium dihydrogen ethylenediaminetetraacetic acid dihydrate	Hach Chemical Co.	
Fructose - 1,6 - diphosphate, Barium salt	Mann Research Laboratories	Chromatographically Pure
Sodium pyruvate	Mann Research Laboratories	99.8% (Titer)
Cytidine 2':3' cyclic phosphate	Schwarz Bio Research, Inc.	

(Hirs, 1953). Sigma Chemical Company designates this preparation of ribonuclease as type II A. All the enzymes were used, as obtained, without further purification. Trypsin and α-chymotrypsin solutions were either 1 mg/ml or 10 mg/ml in either 0.001 M Hcl or tris buffer, the concentration and pH depending on the experiment to be done. The concentrations of trypsin and α-chymotrypsin solutions were determined spectrophotometrically by dividing the absorbance of the solutions in a 1 cm cuvette by their absorptivity coefficients. The absorptivity coefficient for trypsin at 280 mμ is 1.57/cm/mg/ml (Hummel, 1959). For α-chymotrypsin at 282 mμ, it is 1.85/cm/mg/ml (Hummel, 1959). Lactate dehydrogenase solutions were made by diluting the enzyme suspension in 0.01 M NaCl. The pH of 10 mg/ml solutions was 5.8. The exact concentrations of these solutions were determined by dividing the absorptivity, $a^{1\ cm}_{280\ m\mu} = 1.49$/cm/mg/ml (Neilands, 1952), by the $A_{280\ m\mu}$ of the solution. Aldolase solutions were prepared by diluting the enzyme suspension with distilled water to give the desired concentration. The concentration was determined spectrophotometrically using the absorptivity, $a^{0.1\%}_{1\ cm} = 0.91$ (Baranowski and Nieder, 1949), and the measured $A_{280\ m\mu}$ of the solution. Ribonuclease solutions were wither 1 mg/ml or 10 mg/ml in tris buffer or 0.1 M KCl. The exact concentrations of the solutions were calculated from the measured $A_{278\ m\mu}$ of the solution using the absorptivity, $a^{1\ cm}_{278\ m\mu} = 0.715$/cm/mg/ml (Hermans and Sheraga, 1961).

3. Buffer Preparations and pH Measurement

Tris buffer was prepared according to the method of Crook et al. and phosphate buffer according to Gomori, 1955. All pH measurements were made with a Beckman zeromatic pH meter.

B. Enzymatic Activity Assays

All the assays used in these studies were spectrophotometric assays in which formation of reaction products is manifested as a change in the amount of monochromatic light absorbed by the reaction mixture versus time.

Alpha chymotrypsin was assayed by following its esterase activity as described by Schwert and Takenaka, 1955. In this assay, a maximum difference between the absorption spectra of N-acetyl-L-tyrosine ethyl ester (ATEE) and N-acetyl-L-tyrosine occurs at 237 mμ, so that by following the decrease in absorbance as a function of time, the rate of ester hydrolysis can be determined. One unit of α-chymotrypsin activity is equivalent to a change in absorbance at 237 mμ of 0.001 per minute at pH 7.0 and 25°C.

N-acetyl-L-tyrosine ethyl ester was purchased from Worthington Biochemical Corporation in individual tubes ("Determatubes") which, when reconstituted with distilled water, gave a substrate concentration of 0.001 M ATEE in 0.05 M phosphate buffer, pH 7.0. Trypsin was also assayed by following its esterase activity (Schwert, 1955). Observation of the difference in absorbance at 253 mμ between N-benzoyl-L-arginine and its ethyl ester was used to follow the trypsin hydrolysis of the ester. N-benzoyl-L-arginine ethyl ester (BAEE) was obtained from Worthington Biochemical Corporation in "Determatubes" which give, upon adding the appropriate amount of distilled water, 2.5×10^{-4} M BAEE in 0.0667 M phosphate buffer, pH 7.0. A unit of trypsin activity is a change in absorbance at 253 mμ of 0.001 per minute at pH 7.0 and 25°C.

Lactate dehydrogenase (LDH) in the presence of the cofactor reduced nicotinamide-adenine dinucleotide ($NADH_2$) catalyzes the formation of lactate from pyruvate and, in the process of the reaction, the cofactor $NADH_2$ is

oxidized to NAD. The oxidation of the cofactor is accompanied by a marked decrease in its absorption spectrum at 340 mμ which can be utilized to measure the reaction rate. A unit of LDH activity is defined as the catalytic activity which causes an initial rate of oxidation of one micromole of $NADH_2$ per minute at pH 7.4 at 25°C. For assay in a three milliliter cuvette, the enzyme was added to the following mixture: 0.1 ml of 0.01 M sodium pyruvate, 0.1 ml 0.002 M $NADH_2$ whose pH was adjusted to 8.0 with 0.1 M sodium hydroxide, 2.7 ml of pH 7.4, 0.03 M phosphate buffer. The molar absorbancy coefficient for $NADH_2$ in a 1 cm cuvette at 340 mμ is 6.2×10^3 cm^{-1} $mole^{-1}$. Aldolase activity was determined by a modification of the hydrazine assay (Jagannathan, 1956). In this reaction, aldolase catalyzes the cleavage of fructose of -1, 6-diphosphate to dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate and the latter product then reacts with hydrazine to form a hydrazone which absorbs strongly at 240 mμ. The barium salt of fructose -1, 6-diphosphate was converted to the sodium salt by dissolving the barium salt in 0.1 M HCl, adding sufficient sodium sulfate to precipitate the barium as barium sulfate and adjusting the pH to 7.0 with 0.1 M NaOH. The assay system in a 3 ml cuvette consisted of: 2.0 ml of 0.0035 M hydrazine sulfate in 0.0001 M ethylenediaminetetraacetic acid, pH adjusted to 7.5, 1.0 ml of 0.012 M fructose -1, 6-diphosphate (sodium salt). One unit of activity was defined as a change in absorbance of 1.000 per minute at 25°C in the assay system just described. Since FDP reacts to form a hydrazone in the absence of enzyme, it is necessary to run a blank without enzyme and subtract the blank rate from the assay rate to obtain the aldolase catalyzed rate. The assay for ribonuclease activity was one which depends on the difference in the absorbance at 286 mμ of cytidine 2':3'-phosphate and its hydrolysis

product cytidine 3'-phosphate (Crook, 1960). As ribonuclease catalyzes the hydrolysis of cytidine 2':3'-phosphate to cytidine 3'-phosphate, an increase in absorbance at 286 mμ is observed which is proportional to the enzyme's activity. One unit of activity is that which produces an absorbance increase of 0.001 per minute at pH 7.1 and 25°C. The substrate for the assays is 0.2 mg/ml cytidine 2':3'-phosphate in tris buffer.

C. Ultrasonic Irradiation Equipment

1. Equipment for Direct Irradiation of Activity Assays

An instrument designed especially for the purpose of monitoring the ultrasonically irradiated enzyme reactions was constructed to fit a Beckman Model DU spectrophotometer. The cuvette compartment of this spectrophotometer was replaced by an ultrasonic irradiation system consisting of:

a) A 1 MHz X-cut quartz transducer, the piezoelectric element which produces ultrasound when excited electrically;

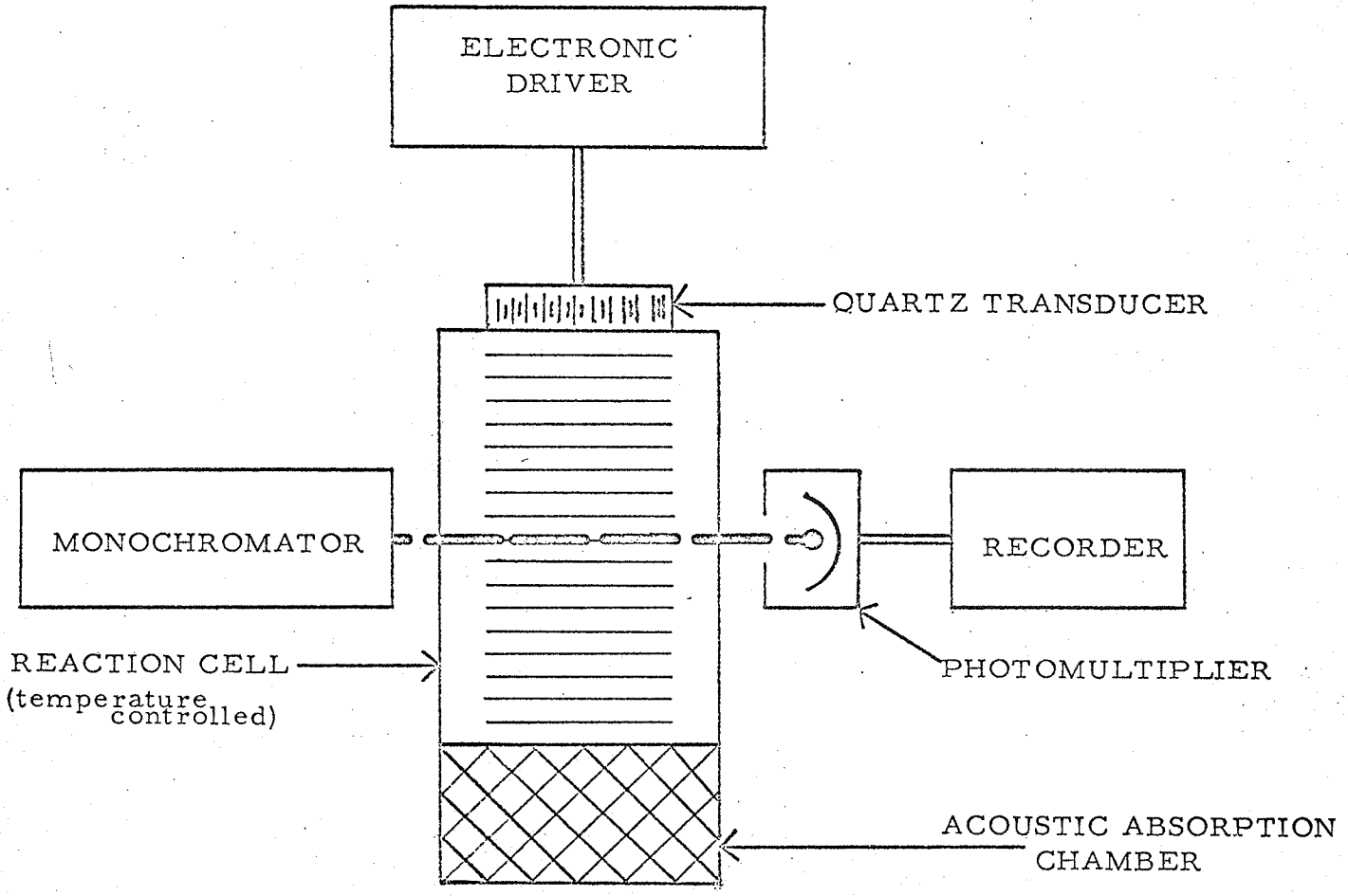
b) A right circular cylindrical reaction cell of stainless steel, volume approximately 20 ml, with quartz windows to transmit the spectrophotometer light beam at right angles to the direction of ultrasonic wave propagation and with water from a constant temperature bath circulating through its walls to maintain a constant temperature; and

c) An acoustic absorption chamber (filled with castor oil and separated from the reaction cell by a 0.001 inch thick polyethylene membrane) whose function is to insure the absence of standing waves by absorbing all incident acoustic energy.

The arrangement of the three components in the spectrophotometer is shown diagrammatically in Figure 2. The U.V. light passing through the

FIGURE 2

ARRANGEMENT OF REACTION CELL FOR ULTRASONIC IRRADIATION IN BECKMAN DU SPECTROPHOTOMETER

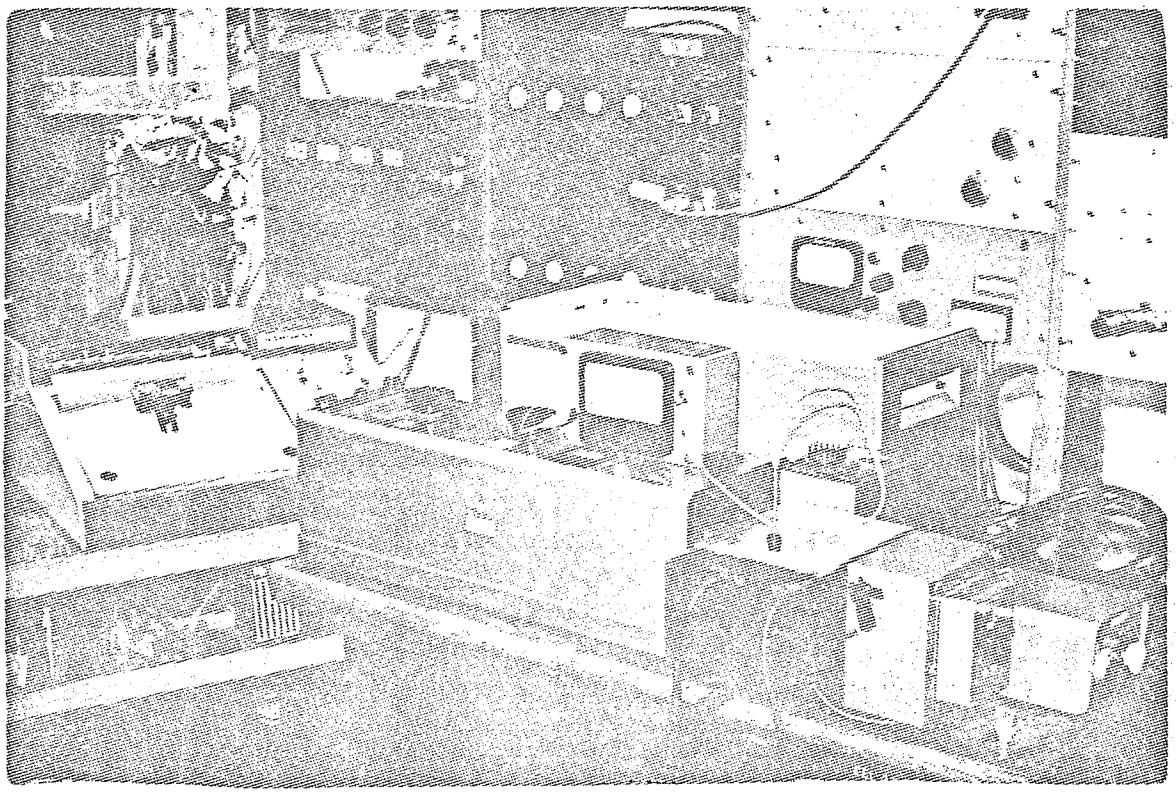


reaction cell is detected by a 1 P28 photomultiplier tube and the electrical output is fed through a Beckman energy recording adapter to a Sargent Model SRL recorder equipped with logarithmic gears to give a plot of absorbance versus time. The slope of the absorbance-versus-time line is the rate of the enzyme catalyzed reaction and can be converted, using the appropriate definition for a unit of activity, to the specific activity of the enzyme. Figure 3 shows photographs of the ultrasonic irradiation system in the spectrophotometer. More detailed specifications and a description of the construction of the system are given in the Appendix. Figures 4 and 5 are block diagrams of the electronic systems used to drive the piezoelectric transducer.

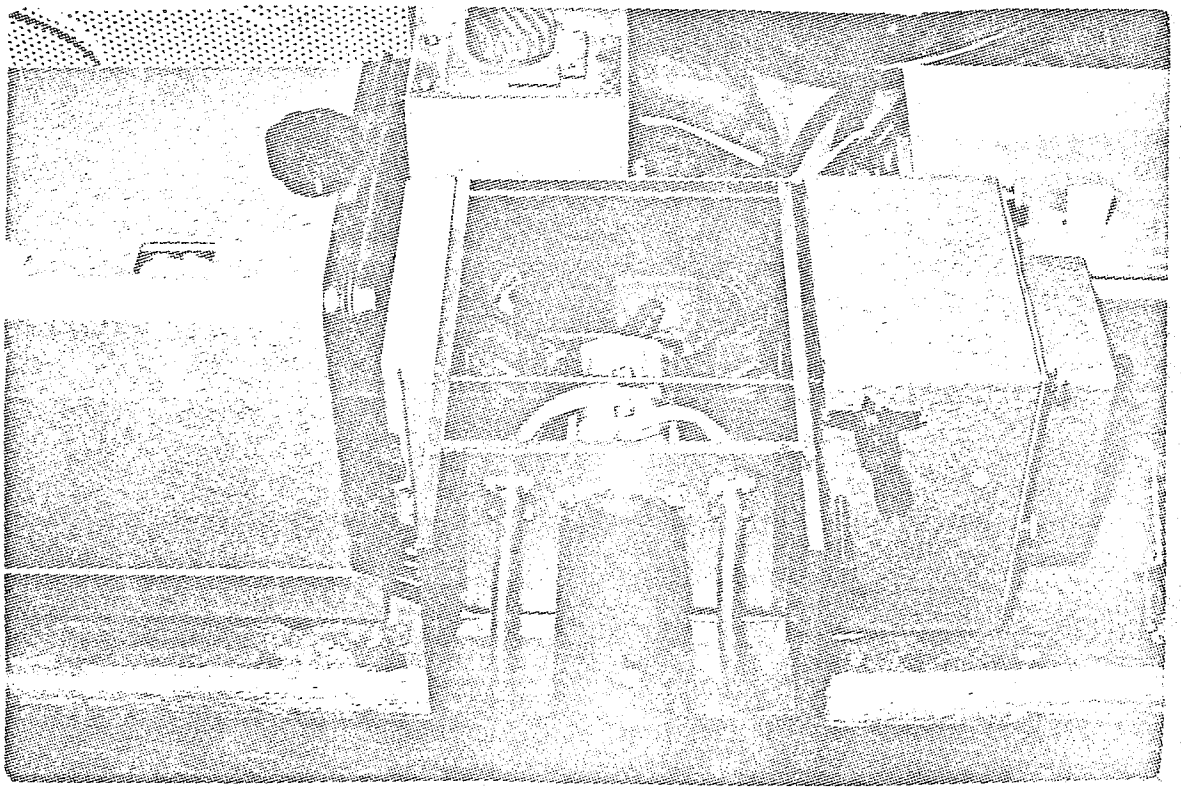
2. Equipment for the Noncavitating, High-Intensity, Ultrasonic Irradiation of Enzyme Solutions

For the study of the effects of intense noncavitating ultrasound on enzyme solutions, two different but basically similar ultrasonic systems were employed, both of which were capable of producing much higher intensities than the system used to irradiate the enzyme catalyzed reactions. One system was arranged for horizontal irradiation, the other for vertical irradiation. Each system consisted of: an X-cut quartz transducer with a fundamental resonant frequency of 1 MHz, coupled by a layer of oil to a plano-concave polystyrene acoustic lens, a large compartment to contain the sound transmitting medium (which was degassed distilled water in all experiments), and an acoustic absorption chamber filled with castor oil and separated from the transmitting medium by a ρc rubber membrane. Each system was also equipped with a mechanical three-dimensional coordinate system which permitted the sample container to be positioned in the focal volume of the sound beam

FIGURE 3



MODIFIED SPECTROPHOTOMETER WITH RECORDER



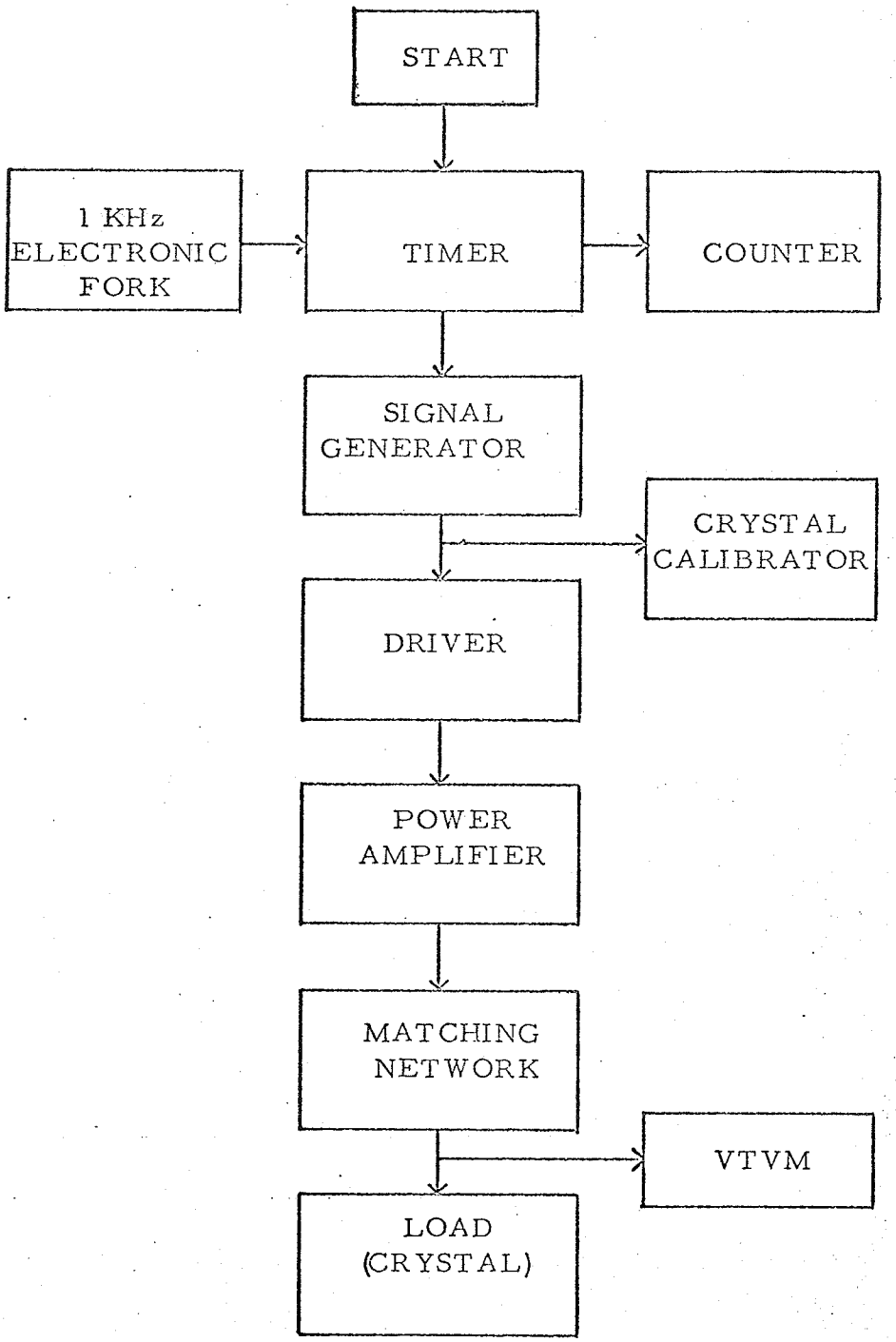
COMPARTMENT IN SPECTROPHOTOMETER

within ± 0.01 cm in each orthogonal direction. In the horizontal system, the transducer is fixed and the sample container is moved by the coordinate system; in the vertical system, the sample has a fixed position and the transducer is movable. The quartz crystal in the horizontal irradiation system was 1 1/2 inch in diameter and was driven by the electronic arrangement diagrammed in Figure 4. At 1 MHz, the focal volume within the sample container was experimentally determined to be approximately 0.5 cm in diameter and 1 cm long. By "focal volume" is meant the volume over which the intensity does not decrease by more than 50 per cent of the peak intensity. The maximum intensity used with this system was usually 75 w/cm^2. The acoustic transmitting medium compartment for the horizontal system contained 12 liters. Although no provision was made to temperature control this compartment, by equilibrating the degassed distilled water to room temperature (usually about $22^{\circ}C$) before siphoning it into the tank, it was found that the temperature never increased more than $0.2^{\circ}C$ (due to increase in room temperature) during an experiment.

In the vertical irradiation system, the quartz crystal was 2 1/2 inches in diameter and was driven by the electronic arrangement diagrammed in Figure 5. At eleven megacycles, the frequency at which this system was usually operated, the focal volume was about 0.05 cm in diameter and about 0.3 cm long. The peak intensity in the focal volume was 1000 w/cm^2. This high intensity requires that irradiation times be short so the acoustic lens will not overheat and alter its focal properties. The timer was set to deliver 0.1 sec pulses of ultrasound and irradiation was automated by arranging a motor driven cam so that it closed a microswitch and keyed the timer once every six seconds. The motor also operated a mechanical counter to record

FIGURE 4

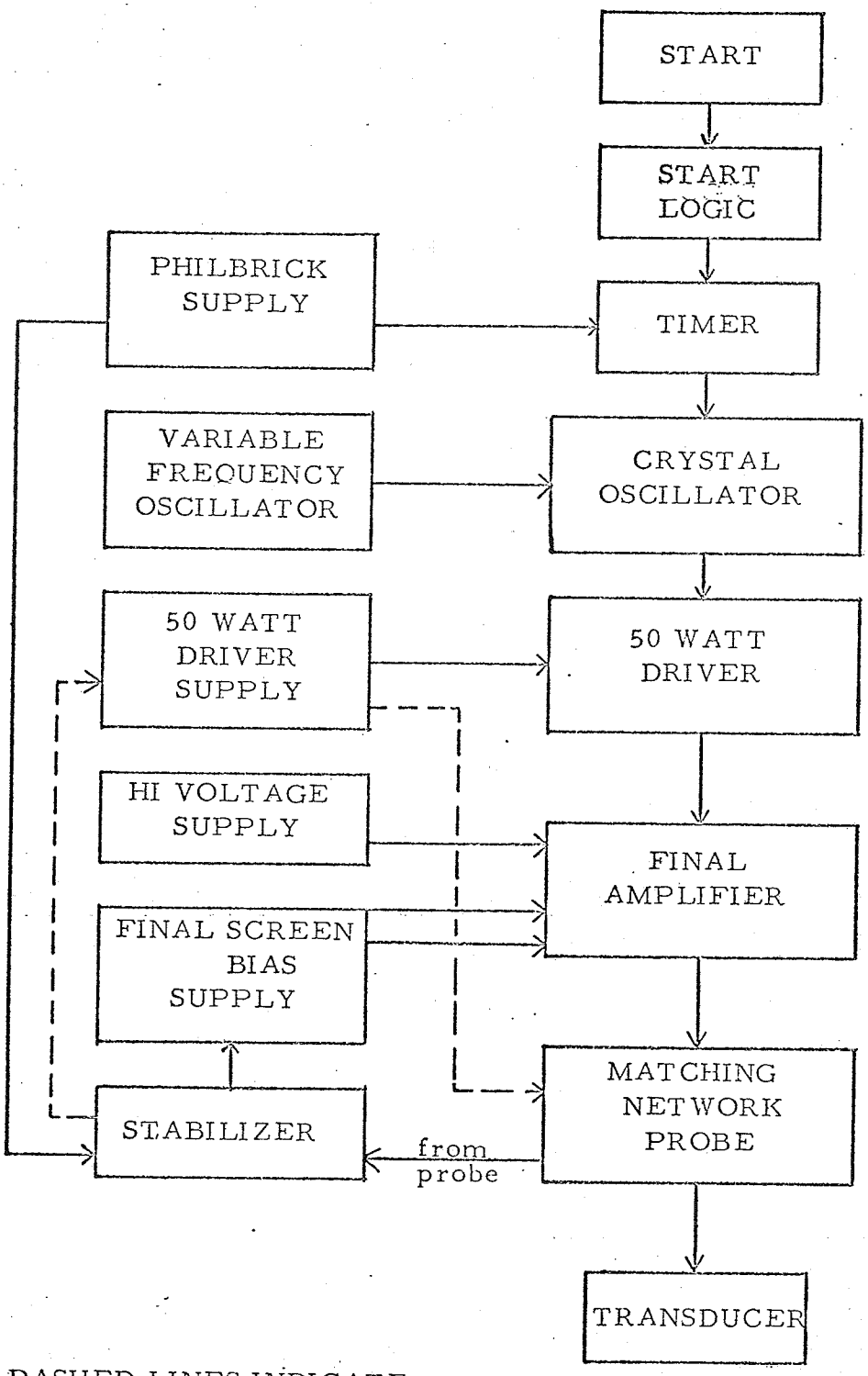
BLOCK DIAGRAM OF ELECTRONICS FOR 1-9 MHz



Crystal Calibrator: Ferris Instrument Co., Model 33-A
Signal Generator: Measurements Corp., Model 80-R
VTVM: Hewlett Packard Co.

FIGURE 5

BLOCK DIAGRAM OF ELECTRONICS FOR 11-27 MHz



DASHED LINES INDICATE
CONNECTIONS FOR LOW LEVEL OPERATION

Timer: Computer Measurements Co.
Variable Frequency Oscillator: Ferris Instrument Co.

the number of ultrasonic pulses delivered. The irradiation process was automated because the combination of small focal volume and short irradiation time required that many pulses be given to insure that an appreciable fraction of the sample be irradiated. The sample size for this system was 2 ml. Experiments with suspensions of small particles showed that a 0.1 sec pulse resulted in energetic particle motion inside the sample container. Thus, since the acoustic pulse provided good stirring and since there was 5.9 sec between pulses, it appears to be a good assumption that complete mixing occurred after each pulse. With this assumption of complete mixing, a conservative estimate of 2 $(mm)^3$ in the focal volume, and a total volume of 2 ml, a simple calculation shows that after 2000 pulses the probability of every particle being in the focal volume at least once is 0.86.

a) Sample Containers: For the horizontal irradiation system, where the focal volume at 1 MHz is about 0.8 ml, sample containers were made of pyrex tubing 32 mm long and 16 mm in I.D. (volume is about 6.4 ml). A groove was ground on the outside of each end of the tube to a depth sufficient to hold a one-sixteenth inch thick "O" ring. Both ends of the tube were closed with pieces of 0.0005 inch thick saran which were clamped in place with the "O" rings. With a little practice, it is possible to fill this container with degassed sample solution and to close it s that air is not trapped inside. For the vertical system, the containers were similar but of lesser volume (2.0 ml). They were made of pyrex tubing of 9 mm I.D. and were 32 mm long. The ends were closed in the manner described above. Both types of containers were clasped in small three-fingered laboratory clamps for mounting. In the horizontal system, the clamp was attached to the mechanical three-dimensional coordinate system for maneuvering. In the vertical system, the clamp was fastened in a fixed position.

b) Location of the Focal Volume and Intensity Determinations: The position of the focal volume in the transmitting medium was located by the thermocouple probe method (Fry and Dunn, 1962; Fry and Fry, 1954). The acoustic intensity in the focal volume of the horizontal system was determined by the radiation pressure technique (Fry and Dunn, 1962). The vertical irradiation system had already been calibrated so that the intensity in the focal volume of this system was already known.

3. Cavitation Equipment

Most of the experiments with cavitating ultrasound were carried out using a Branson Sonifier, model LS-75, equipped with a microtip. The operating frequency of this instrument is 20 kilocycles. All experiments were done at power setting number 2. The instrument has eight power settings with the highest number corresponding to the highest power. At power settings higher than two, the cavitation was so vigorous that much of the sample was splashed out of the container even though it was partially covered (see below). 4 ml samples were irradiated (cavitated) in stainless steel Morton culture tube closures (17.5 mm I.D. × 37.5 mm long) covered with parafilm with a hole provided through which the microtip was inserted into the sample solution. Since considerable heat is generated in a cavitating soluti n, the containers were kept immersed in an ice water bath at $0^{o}C$ and irradiation times were short. For example, if the total irradiation time for a sample was to be 30 minutes, it would be given in ten 3-minute irradiations during which the temperature was always below $20^{o}C$ and cooling down to $2^{o}C$ was allowed between the 3-minute exposures. Temperatures were measured within 5 seconds after an irradiation with a thermistor probe and a Tele-Thermometer.

A set of experiments was carried out at 1 MHz in the high-intensity horizontal irradiation system described in Section C, 2. The samples were thoroughly aerated and placed in the containers described for this system. In filling, bubbles of air were purposely introduced before closing the containers. Cavitation was observed when the peak intensity was above 100 w/cm^2 although it was not as vigorous as that produced at 20 kHz by the Branson equipment. The temperature of the samples during the irradiations was 27.3°C.

D. Analytical Equipment and Method

1. Spectrophotometric Enzyme Activity Assays

All the enzymes studied were assayed for activity by the spectrophotometric methods given above. The reactions were followed with a Beckman DU spectrophotometer equipped with a deuterium lamp ultraviolet light source, thermostated cuvette compartment and a photomultiplier detector whose output was adapted to a Sargent Model SRL recorder by means of a Beckman Energy Recording Adapter. The recorder had logarithmic gears so that a direct plot of the reaction rate (absorbance versus time) was obtained. Three millimeter volume, fused silica cuvettes were used for the assays. All reactions were run with a cuvette compartment temperature of 25.0 ± 0.2°C. The recorder, with logarithmic gears in place, was calibrated and found to give true logarithmic output over its whole range to better than 1 per cent.

2. Ultraviolet Absorption Spectra

Ultraviolet absorption spectra in the wavelength range 320 mμ to 240 mμ were read either with the Beckman DU used for activity assays (without the recorder) or with a Cary Model 14 automatic scanning and recording spectrophotometer versus a solvent blank. The absorptivity, a, of an absorbing

substance is defined by

$$a = \frac{A}{b\,c} \qquad (16)$$

where A is the measured absorbance of a solution of the substance defined by $A = \log_{10} (I_o/I)$, where I_o is the intensity of the light incident on the solution, and I is the intensity of the light transmitted through the solution; b is the length of the light path in the solution; and c is the concentration of the absorbing substance. The b in all measurements was 1 cm. When the molecular weight of the absorbing substance was known, c is expressed in moles per liter and a is the molar absorptivity. For the case where the molecular weight is unknown, c is expressed in grams per cubic centimeter and absorptivity has the units of centimeters squared per gram or centimeters per milligram per milliliter. For proteins whose molecular weight is not exactly known, values of a for 1 per cent solutions measured in a 1 cm cuvette were found in the technical literature.

3. Specific Optical Rotation

Optical rotation measurements were made with a Rudolph Model 70 Precision Polarimeter using a 10.00 cm microtube (0.7 ml) and illuminated with a sodium vapor lamp. The specific rotation, $[\alpha]_D^{T^oC}$, for the sample being measured was calculated using the relation:

$$[\alpha]_D^{T^oC} = \frac{\alpha_{obs}}{\ell \times c} \qquad (17)$$

where α_{obs} is the observed rotation, ℓ is the length of the light path in the solution in decimeters, and c is the concentration of the substance being measured in grams per cubic centimeter. The subscript D in $[\alpha]_D^T$ denotes that the "D" line of sodium (589 mμ) is the wavelength of the light source. The

superscript T is the temperature, in degrees Centigrade, of the solution when measured. α_{obs} of sample solutions were corrected for any rotation due to their solvent before calculating $[\alpha]_D^T$ using (17).

4. Sedimentation Velocity Analysis

All sedimentation velocity analyses were done on a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics (phase plate Schlieren diaphragm), a rotor temperature indicator and control (RTIC) unit. An An-D rotor was used in all experiments. Both the standard cell with a Kel-F center piece and a valve type synthetic boundary cell were employed. When a sample solution and its control were run simultaneously, the standard cell containing the sample had a 2^o positive wedge window which displaced the Schlieren image for the sample solution to the upper half of the photographic plate. The Schlieren patterns were photographed on Kodak Metallographic plates and the images were measured with a Bausch and Lomb toolmaker's microscope which could be read to 0.0001 inch in two orthogonal directions. Rough measurements were made by the method described by Markham (1960).

The observed value of the sedimentation coefficient $S_{T,b}$ (at temperature T in buffer b) was calculated from the equation (Svedberg and Pedersen, 1940a)

$$S_{T,b} = 2,303 \frac{\log r/r_o}{\omega^2(t - t_o)} \qquad (1.)$$

where r is the distance of the boundary from the center of rotation in centimeters, t is the time in seconds, ω is the angular velocity of the rotor in centimeters per second, and r_o and t_o refer to the values of r and t when the first photograph was taken. The observed values were converted to standard conditions of 20^oC and water as the solvent by the

following equation:

$$S_{20,w} = S_{T,b}\left(\frac{\eta_{T,w}}{\eta_{20,w}}\right)\left(\frac{\eta_b}{\eta_w}\right)\left(\frac{1 - \bar{v}_{20}\,\rho_{20,w}}{1 - \bar{v}_T\,\rho_{T,b}}\right) \qquad (19)$$

where η is the viscosity, v is the partial specific volume of the enzyme, ρ is the density of the solvent and the subscript w refers to water.

The variation of viscosity of water with temperature (the first viscosity term) was obtained from the data of Svedberg and Pedersen (1940b). η_b/η_w was either taken from Svedberg and Pedersen (1940c), estimated by linear extrapolation from this source or, in the case of tris buffer, was determined experimentally using a Cannon-Ubbelhode No. 50 semi-micro dilution viscometer at 20.00 ± 0.02°C. Solvent densities were measured using a pycnometer and a semi-micro balance (Ainsworth Model 10) having a least count of one-tenth of a milligram or were taken from data in the "Handbook of Chemistry and Physics" (1961 edition). No correction was made for partial specific volume since the temperature did not differ by more than 2°C from 20°C and the temperature coefficient for the enzymes of this report is such that v changes with temperature by about one part in 750 per degree Centigrade (Svedberg and Pedersen, 1940c).

5. Chromatography

Thin-layer chromatography experiments were performed using the Eastman Chromagram Developing apparatus and Eastman Chromagram Sheets, Type K301R. The pre-coated sheets are 20 cm x 20 cm poly (ethylene terephtalate), coated with a 100 μ thick layer of silica gel containing a fluorescent indicator. Samples were spotted with lambda pipettes. The solvent system used was n-butanol; acetic acid; H_2O (3:1:1). After development and drying, spots

were located first by examining under ultraviolet light and then by spraying with ninhydrin, 0.3 per cent in absolute ethanol, and heating at 90°C for 15 minutes.

E. Degassing Procedure

Below ten megacycles the acoustic intensity cavitation threshold for water containing dissolved gas is lower than for degassed water (Fry and Dunn, 1962a). Data for the acoustic intensity cavitation threshold for most aqueous solutions is not available but the general principle that the threshold increases with viscosity (Fry and Dunn, 1962c) means that the data for water can be considered minimum values for aqueous solutions more viscous than water. Thus, to avoid cavitation for ultrasonic frequencies below ten megacycles, the sample solution should be degassed and the intensity kept below the acoustic cavitation threshold for degassed water.

Degassing was carried out in a vacuum desicator using a laboratory vacuum pump. The sample solution, seven to twenty milliliters depending on the sample container size and the number of samples, was placed in a fifty milliliter erlenmeyer flask and the flask was covered with a piece of perforated parafilm to prevent loss of sample due to the splashing and foaming which occur during degassing. The flask was then placed in the vacuum desicator and the vacuum was applied for ten minutes. All the visibly detectable degassing was usually over within five minutes. After ten minutes under vacuum, the desicator was slowly brought to atmospheric pressure, the sample solution was removed and the volume lost due to evaporation of water was replaced by bringing the solution back to its original volume by carefully adding degassed distilled water.

III. EXPERIMENTAL

A. Effects of Intense Noncavitating Ultrasound on Enzymes in Solution: Irradiations and Analyses

The composition of the enzymes solutions studied, the characteristics of the ultrasound to which they were exposed, and the analyses of the irradiated solutions are given in Tables 4,5,6 and 7. Preliminary experiments showed that about 10 mg/ml was the best enzyme concentration for these studies. At concentrations less than 5 mg/ml, there was insufficient material in the 2.0 ml samples to carry out all the analyses, particularly the specific optical rotation determinations. At concentrations from 10 to 12 mg/ml, enzyme precipitated from some solutions during the degassing procedure and surface denaturation while filling the sample containers became a problem.

The solutions were degassed according to the procedure given in II, E and the sample containers were filled and mounted in the focal volume of the ultrasonic irradiation systems as described in II, C, 2. In each experiment, a second container was filled and placed in the transmitting medium compartment, but out of the sound beam, to serve as a control.

Following irradiation, samples and controls were refrigerated at 5°. until the various analyses could be performed. Appropriate dilutions of aliquots of the irradiated samples and controls were assayed for enzymatic activity by the methods described in II, D, 1. The results of the other analyses in Tables 4, 5, 6 and 7 were obtained by the methods given in II, D: 2, 3 and 4.

The results of the analyses presented in Tables 4, 5, 6 and 7 show that

TABLE 4

ALPHA CHYMOTRYPSIN

	1	2	3	4
ENZYME ENVIRONMENT				
Conc. (mg/ml)	10.20	10.48	9.62	10.32
Solvent	tris buffer	pH 3, 0.1 M NaCl	pH 3, 0.1 M NaCl	tris buffer
pH of Solvent	7.1	3.0	3.0	7.1
Temperature (^{o}C)	26.4	23.8	37.0	37.0
ULTRASONIC TREATMENT				
Freq. (MHz)	1	11	11	11
Intensity (w/cm^{2})	75	1000	1000	1000
Duration	10 min. continuous	1800 0.1 sec. pulses	2000 0.1 sec. pulses	2000 0.1 sec. pulses
ANALYSES				
Specific Activity (U/mg)				
Sample	15,300	13,500	11,100	9300
Control	15,300	13,500	11,100	9300
$A_{280\ m\mu}$ (of 1:100 dil.)				
Sample	0.185	0.191	0.177	0.191
Control	0.185	0.191	0.176	
$A_{280\ m\mu}/A_{250\ m\mu}$				
Sample	2.83	2.65	2.60	2.55
Control	2.83	2.65	2.67	
$[\alpha]_{D}$ (at T^{o}C)				
	(22^{o}C)	(24^{o}C)	(25^{o}C)	
Sample	- 54^{o}	- 52^{o}	- 53^{o}	
Control	- 45^{o}	- 48^{o}	- 47^{o}	
$S_{20,w} \times 10^{13}$ (sec.)				
Sample	2.63			2.37
Control	2.65			2.58

TABLE 5

TRYPSIN

	1	2	3
ENZYME ENVIRONMENT			
Conc. (mg/ml)	9.05	9.24	8.40
Solvent	tris buffer	pH 3, 0.1 M NaCl	pH 3, 0.1 M NaCl
pH of Solvent	7.1	3.0	3.0
Temperature (^{o}C)	20.2	22.7	37.0
ULTRASONIC TREATMENT			
Freq. (MHz)	1	11	11
Intensity (w/cm^{2})	75	1000	1000
Duration	10 min. continuous	2000 sec. 0.1 pulses	2000 sec. 0.1 pulses
ANALYSES			
Specific Activity (U/mg)			
Sample	4600	5200	3800
Control	4600	5200	3800
$A_{280\ m\mu}$ (of 1:100 dil.)			
Sample	0.142	0.145	0.132
Control	0.141	0.145	0.132
$A_{280\ m\mu}/A_{250\ m\mu}$			
Sample	2.60	2.50	2.60
Control	2.62	2.59	2.64
$[\alpha]_{D}$ (at T^{o}C)	(22^{o}C)	(24^{o}C)	(23^{o}C)
Sample	-23^{o}	-48^{o}	-56^{o}
Control	-24^{o}	-55^{o}	-62^{o}
$S_{20,\ w} \times 10^{13}$ (sec.)			
Sample	1.80		1.41
Control	1.54		1.46

TABLE 6

LACTATE DEHYDROGENASE

ENZYME ENVIRONMENT	1	2
Conc. (mg/ml)	8.19	11.20
Solvent	~ 17% sat. $(NH_4)_2 SO_4$	~ 17% sat. $(NH_4)_2 SO_4$
pH of Solution	5.8	5.8
Temperature (^oC)	23.0	23.7
ULTRASONIC TREATMENT		
Freq. (MHz)	1	11
Intensity (w/cm^2)	75	1000
Duration	10 min. continuous	2000 0.1 sec. pulses
ANALYSES		
Specific Activity (U/mg)		
Sample	34	29
Control	34	29
$A_{280\ m\mu}$ (of 1:100 dil.)		
Sample	0.122	0.167
Control	0.122	0.166
$A_{280\ m\mu}/A_{250\ m\mu}$		
Sample	2.60	1.25
Control	2.54	1.29
$[\alpha]_D$ (at T^oC)	(22^oC)	(24^oC)
Sample	-43^o	-38^o
Control	-37^o	-36^o
$S_{20,w} \times 10^{13}$ (sec.)		
Sample	5.40	
Control	5.41	

TABLE 7

ALDOLASE AND RIBONUCLEASE

	ALDOLASE	RIBONUCLEASE
ENZYME ENVIRONMENT		
Conc. (mg/ml)	11.00	9.22
Solvent	~ 25% sat. $(NH_4)_2 SO_4$	0.1 M Kcl
pH of Solvent	7.8	6.8
Temperature (^oC)	24.4	24.2
ULTRASONIC TREATMENT		
Frequency (MHz)	11	11
Intensity (w/cm^2)	1000	1000
Duration	2000 0.1 sec. pulses	2000 0.1 sec. pulses
ANALYSES		
Specific Activity (U/mg)		
Sample	11	1100
Control	11	1100
$A_{280\ m\mu}$ (of 1:100 dil.)		
Sample	0.100	0.062
Control	0.100	0.063
$A_{280\ m\mu}/A_{250\ m\mu}$		
Sample	3.00	2.10
Control	2.94	2.14
$[\alpha]_D$ (at T^oC)	(23^oC)	(24^oC)
Sample	-20^o	-53^o
Control	-18^o	-68^o
$S_{20,\ w} \times 10^{13}$ (sec.)		
Sample	6.26	
Control	6.32	

there are no significant differences between the irradiated samples and their unirradiated controls in any of the experiments and, thus, the enzymes are unaffected in any permanent way be the intense noncavitating ultrasound. A more detailed consideration of each table follows.

Alpha chymotrypsin is known to be most stable in solution at pH 3 to 4 and at temperatures in the range $0^{o}C$ to $5^{o}C$; α-chymotrypsin solutions of higher pH and solutions stored at higher temperatures undergo gradual autolysis (Kunitz and Northrup, 1935). These facts explain why the specific activities observed for both sample and control in experiments 3 and 4 of Table 4 are lower than the specific activities for the controls at the same pH's but lower temperatures in experiments 1 and 2. Additional control samples in experiment 3 (pH 3.0) and 4 (pH 7.1) which were degassed but not subjected to the 3 1/3 hours (time required for irradiation) at $37^{o}C$ showed the same specific activities as the controls for experiments 2 (pH 3.0) and 1 (pH 7.1), respectively. The interesting conclusion drawn from these results is that α-chymotrypsin is not affected by intense noncavitating ultrasound even under conditions which cause gradual denaturation of the enzyme.

The $A_{280\ m\mu}$ and the ratio $A_{280\ m\mu}/A_{250\ m\mu}$ for 1:100 dilutions of irradiated sample and control are given in Table 4 in order to compare the absorption spectra. In experiments 1, 2 and 3, the sample spectrum was essentially congruent to that of the control and the congruence is reflected in the near identity of the $A_{280\ m\mu}$ and $A_{280\ m\mu}/A_{250\ m\mu}$ values. Only an activity assay and sedimentation velocity analysis were performed on the control for experiment 4 because most of this solution was lost accidentally.

In experiments 1, 2 and 3, the specific optical rotations of the irradiated samples consistently showed slightly more levorotation than the controls.

A value reported in the literature of $[\alpha]_D$ for α-chymotrypsin in 0.1 m NaCl, pH 3 is $[\alpha]_D = -66^\circ$ (Schellman, 1958). The discrepancy between Schellman's value and the values for the control in Table 4 is probably partly due to the difference in the method used for determining concentration. Schellman determined concentrations by a micro-Kjeldahl method assuming the α-chymotrypsin to be 16.06 per cent nitrogen; the concentrations in Table 4 were determined spectrophotometrically using an absorptivity at 282 mμ for α-chymotrypsin of 1.85/cm/mg/ml (Hummel, 1959).

Schwert and Kaufman measured α-chymotrypsin concentrations by the micro-Kjeldahl method assuming a nitrogen content of 15.83 per cent and determined an absorptivity at 282 mμ for α-chymotrypsin of 2.075/cm/mg/ml (Schwert and Kaufman, 1951). Since assuming a larger nitrogen content, as Schellman did, will result in a smaller concentration for identical Kjeldahl determinations, adjusting the value 2.075/cm/mg/ml to conform to Schellman's assumed nitrogen content will give an absorptivity at 282 mμ of 2.10/cm/mg/ml. Using this value to adjust concentrations in Table 4 will change the concentration by the factor 1.85/2.01 and the resulting concentration decrease will result in larger levorotation values for $[\alpha]_D$. For example, an $[\alpha]_D$ which was -47° becomes -53° and -52° becomes -60°. Unless a larger concentration "correction" is used, the values in Table 4 are still less levorotatory than the value reported by Schellman. However, it is not unusual for $[\alpha]_D$ values reported by different investigators for proteins under similar conditions to differ slightly.*

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* See Table VII, pp. 486-489 in Urnes and Doty (1961).

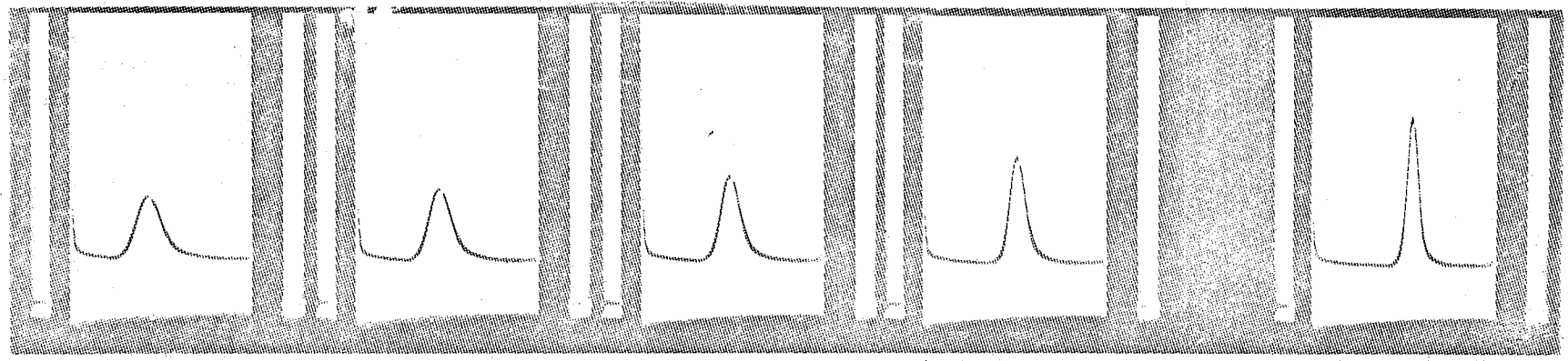
When proteins are denatured, they usually show a large increase in levorotation. If α-chymotrypsin is denatured with 8 M urea in 0.1 M NaCl, pH 3, $[\alpha]_D$ = - 111.6 (Schellman, 1958). Therefore, though the small differences between the specific rotations of samples and controls in Table 4 were consistently found, because they are small differences they are not interpreted as evidence of denaturation.

Sedimentation velocity analysis was done in experiments 1 and 4. In experiment 1, the irradiated sample and control, which were dialyzed versus cold 0.1 M NaCl (pH 6.5) before sedimentation, display identical sedimentation behavior; not only are the sedimentation coefficients nearly equal, but the schlieren patterns are congruent (Figure 6). The value of $S_{20,w}$ found is a reasonable one for the pH and concentration of the solutions (Schwert, 1949). In experiment 4, sufficient material was recovered from the accident which befell the control so that its sedimentation coefficient could be determined and compared to the sedimentation coefficient of the irradiated sample. These determinations were carried out in tris buffer, pH 7.1. The results are consistent with the established sedimentation behavior of native α-chymotrypsin; that is, in the absence of dimer formation, the control, with a concentration at least 10 fold less than the sample, is expected to have a slightly greater sedimentation coefficient than the sample and the sedimentation coefficient of the sample at pH 7.1 is less than the sedimentation coefficient of the sample (at approximately the same concentration) found in experiment 1 at pH 6.5 (Schwert, 1949).

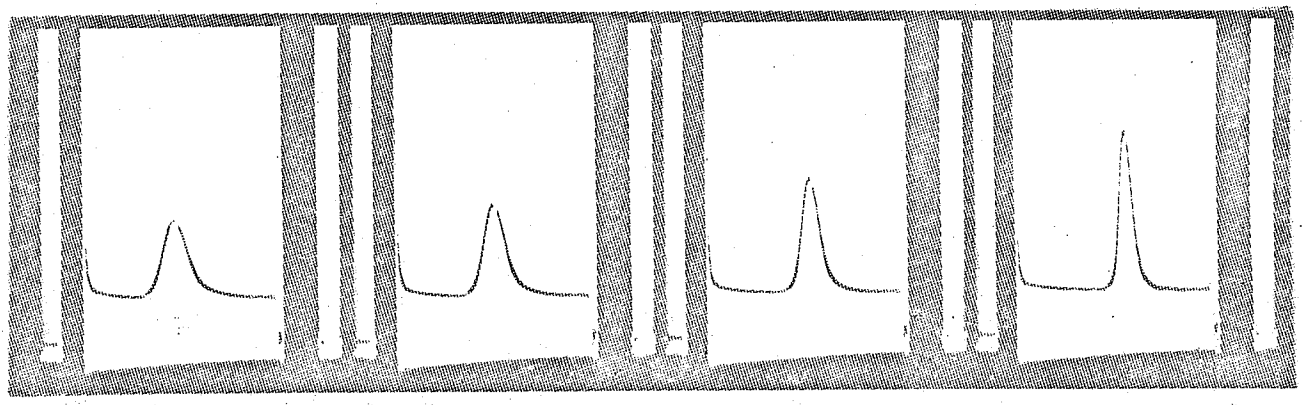
The specific activities and U.V. absorption spectra of the irradiated samples are identical to those of the controls in all experiments on trypsin (Table 5). The specific optical rotations of samples and controls do not

FIGURE 6

ALPHA CHYMOTRYPSIN SEDIMENTATION VELOCITY PATTERNS



SAMPLE (TABLE 4, EXPERIMENT 1)



CONTROL (TABLE4, EXPERIMENT 1)

differ significantly and since trypsin is reported to have a $[\alpha]_D = -40^\circ$ at pH 5.2 and a $[\alpha]_D = -69^\circ$ at pH 1.3, the values at pH 7.1 and pH 3 given in Table 5 seem reasonable. The sedimentation coefficients for experiments 1 and 3 of Table 5, though approximately equal, are not characteristic of native trypsin. The $S_{20,w}$ for 1 per cent trypsin in tris buffer should be approximately 2.35 and for 1 per cent trypsin in 0.1 M NaCl, pH 3, $S_{20,w}$ should be approximately 2.45 (Cunningham et al., 1953). The low $S_{20,w}$ value has been reported before (Nord and Bier, 1953), and is attributed to changes in molecular size due to autolysis. The changes in size, however, do not make trypsin susceptible to damage by intense noncavitating ultrasound since the controls were essentially the same as the irradiated samples. In experiment 3, exposure to a temperature of 37°C lowered the specific activity of trypsin but did not greatly affect the other properties of the enzyme. This was shown by analysis of a second control for the irradiation of experiment 3 which was degassed but not subjected to the 3 1/3 hours at 37°C. The results of the analyses on this "degassed only" control, for comparison to experiment results in Table 5, are:

$$\text{Specific Activity} = 5950 \text{ U/mg}$$

$$A_{280\ m\mu} \text{ (of 1:100 dil.)} = 0.132$$

$$A_{280\ m\mu}/A_{250\ m\mu} = 2.68$$

$$[\alpha]_D^{23^\circ C} = -58^\circ$$

$$S_{20,w} = 1.53\ S$$

The heated control and heated irradiated sample each have 64 per cent of the specific activity of this control and, thus, as with α-chymotrypsin, intense noncavitating ultrasound did not affect the enzyme even in an environment which gradually denatures it. Figure 7 shows the sedimentation velocity diagrams

FIGURE 7

TRYPSIN SEDIMENTATION VELOCITY PATTERNS

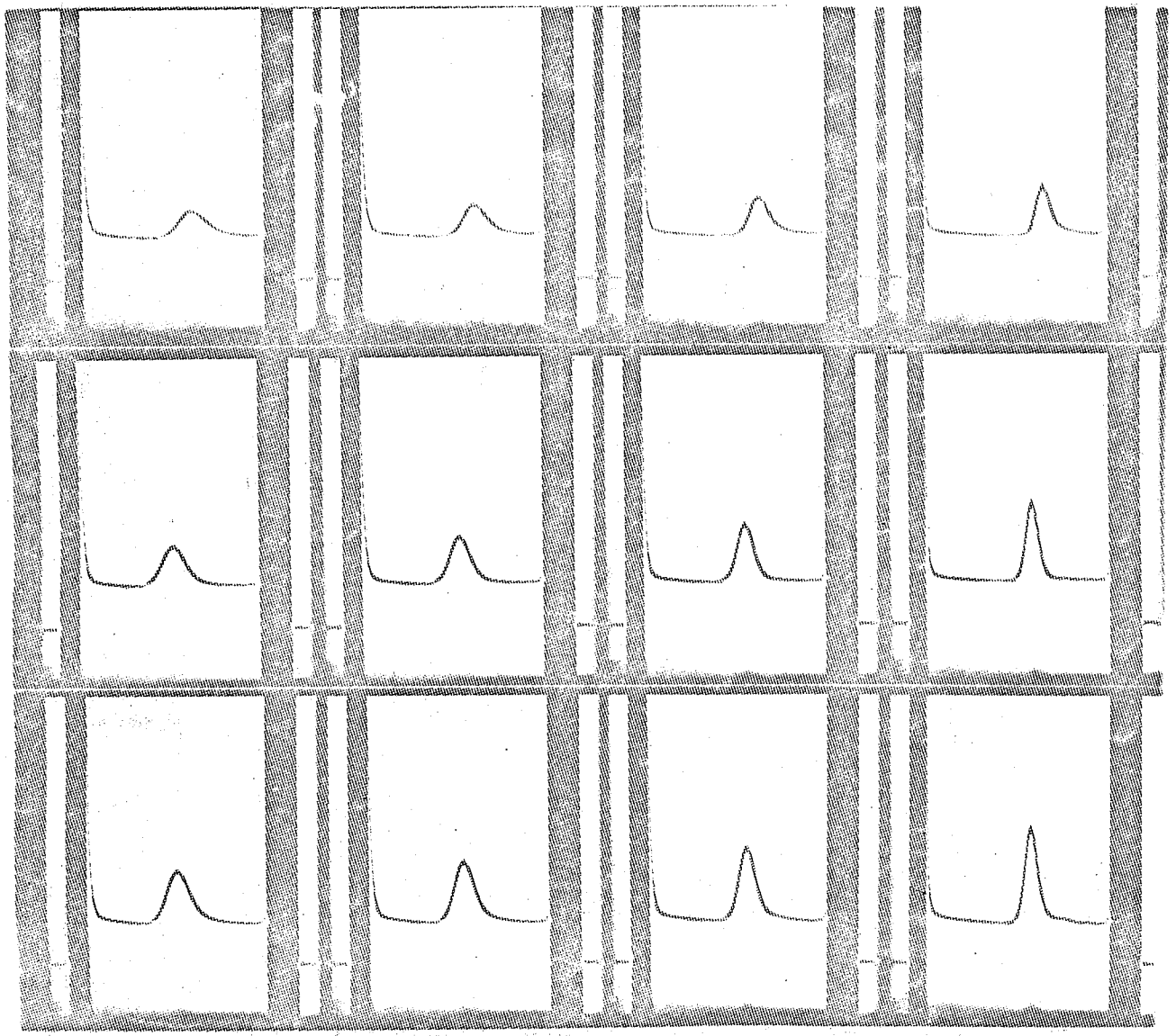


TABLE 5,
EXPERIMENT 3

SAMPLE
(4.24 mg/ml)

HEATED
CONTROL
(5.27 mg/ml)

DEGASSED
ONLY
CONTROL
(6.98 mg/ml)

for experiment 3. The boundary is asymmetric to the slower sedimenting side of the peak and broadens during sedimentation in each case. This indicates that the enzyme solutions were heterogeneous, as would be expected if autolysis occurred.

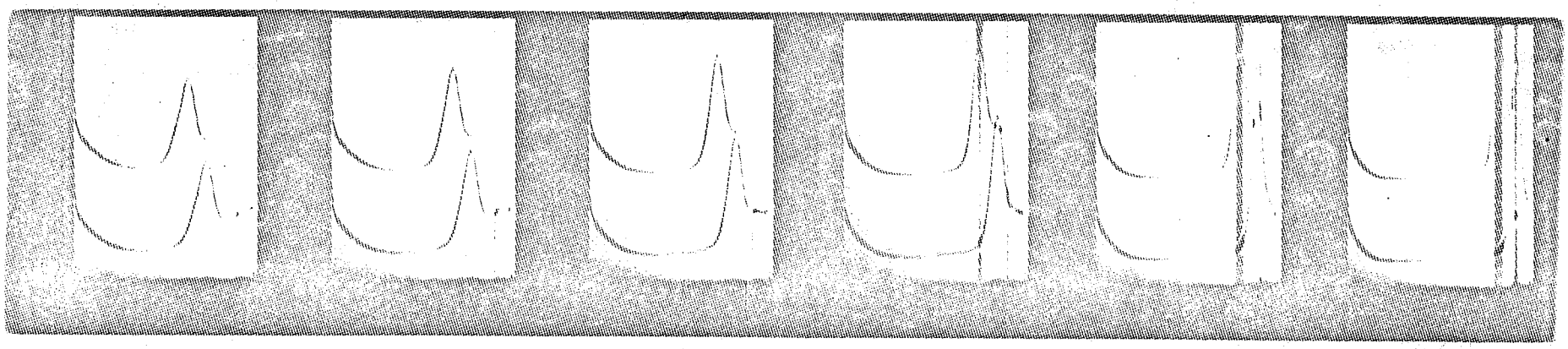
The specific activities and U.V. absorption spectra for irradiated LDH samples and their controls were identical for both experiments in Table 6. The irradiated sample and control of experiment 2 were slightly turbid. This turbidity caused an abnormally high absorption in the 240 to 260 mμ region of the U.V. absorption spectra which in turn yielded low values for the $A_{280\ m\mu}/A_{250\ m\mu}$ ratios (Table 6). The turbid solutions were centrifuged at 24,000 g for 10 min. to obtain clear solutions for optical rotation studies. The specific optical rotation results of Table 6 are close to a reported value of -43^{o} for LDH in pH 5.6, 19% saturated $(NH_4)_2\ SO_4$ (Jirgensons, 1959).

The irradiated sample and control of experiment 1 of Table 6 show the same sedimentation behavior (Figure 8a). The solutions were sedimented in 17% saturated $(NH_4)_2\ SO_4$ and, at this high salt concentration, LDH is dissociated into two subunits each of molecular weight ~ 72,000 (Millar, 1962). The $S_{20,w}$ values found for LDH subunits (at a concentration of 8.19 mg/ml) are in agreement with Millar's value of $S_{20,w} = 5.5$ (at a concentration of 2.6 mg/ml) for, if the $S_{20,w}$ of the subunits decreases with increasing concentration (as does undissociated LDH), then an $S_{20,w}$ at 8.19 mg/ml should be slightly lower than one for a 2.6 mg/ml solution (Millar, 1962).

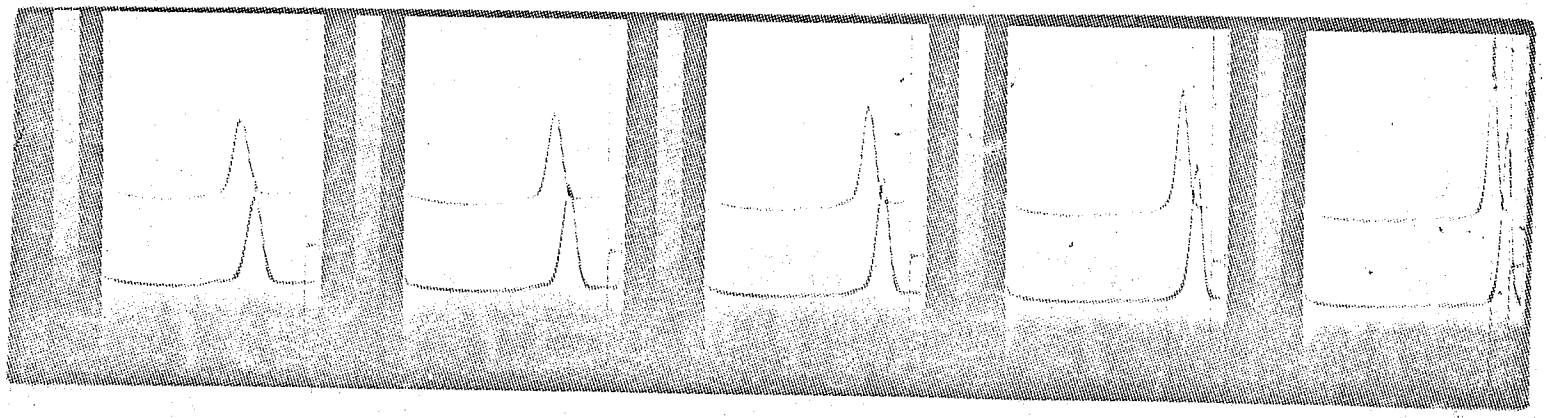
Table 7 contains the results of 11 MHz irradiations of aldolase and ribonuclease. The irradiated aldolase sample and its control have identical specific activities and U.V. absorption spectra. The specific rotations are very nearly equal and are close to a reported value, $[\alpha]_D = -23^{o}$ (Stellwagen

FIGURE 8

SEDIMENTATION VELOCITY PATTERNS



(a.) LDH: TABLE 6, EXPERIMENT 1 : SAMPLE, TOP; CONTROL, BOTTOM



(b.) ALDOLASE: TABLE 7 : SAMPLE, TOP; CONTROL, BOTTOM

and Schachman, 1962). The sedimentation behavior of irradiated sample and control (both dialyzed versus 0.1 M NaCl, pH 6.5 prior to sedimentation) are also practically identical (Figure 8b) but the sedimentation coefficients are lower than the value 6.80 S (Taylor and Lowry, 1956) expected for the concentration and pH of the solutions indicating the possibility of a slight expansion of the aldolase molecules. Such an expansion could have occurred since the sample and control were held at 24.4°C for 3 1/3 hours during the irradiation. That partial denaturation does occur in the sample and control was shown by analysis of the native solution and a control which was immediately refrigerated after degassing. The results of these analyses are:

	Degassed only Control	Native
Specific Activity	12 U/mg	13 U/mg
$A_{280\ m\mu}$ (1:100 dil.)	0.100	0.104
$A_{280\ m\mu}/A_{250\ m\mu}$	3.07	3.08
$S_{20,w}$	6.48 S	6.50 S

During degassing, surface denaturation was observed to cause a small amount of flocculent precipitate. The precipitate removed some protein from solution and this is why the degassed only control, heated control and irradiated sample are slightly less concentrated than the native solution (see $A_{280\ m\mu}$ results). The surface denaturation also accounts for the specific activity of the degassed only control being lower than that of the native solution. The solutions held at 24.4°C for 3 1/3 hours have a lower specific activity than the degassed only control as well as lower sedimentation coefficients but, as with previously discussed enzymes, although aldolase is unstable in

the irradiation environment it still was not damaged by intense noncavitating ultrasound.

The results for ribonuclease in Table 7, with the exception of the specific optical rotation of the irradiated sample, all indicate that the irradiated sample and control solutions were identical. The $[\alpha]_D = -68^o$ observed for the ribonuclease control agrees well with the value -73.3^o reported by White (1961). Explaining the $[\alpha]_D$ of the irradiated sample is difficult because all reported denaturations of ribonuclease, e.g., by oxidation, reduction, high pH, or 8 M urea,* give substantial increases in levorotation and this sample exhibits a decrease in levorotation. Ribonuclease is known to form enzymatically active aggregates (Crestfield et al., 1962) but no reports are available on the optical rotatory behavior of the aggregated material. If aggregates form under the influence of ultrasound and if aggregation leads to a decrease in levorotation, the result observed would be explained. Before the sample could be examined for high molecular weight material by ultracentrifugation, a mold developed in the sample and the experiment was not repeated.

Since some of the solutions of Tables 4, 5, 6 and 7 were dialyzed prior to sedimentation velocity analysis, it was necessary to determine if amino acids or small peptides were cleaved from the enzyme molecules, either by the ultrasonic treatment or, in the case of trypsin and α-chymotrypsin, by autolysis. Thus, some of the irradiated samples and controls were analyzed by thin-layer chromatography according to the methods outlined in II, D, 5. The limit of detection for most amino acids by thin-layer chromatography is lower than 0.1 μg (Randerath, 1965). Aliquots of the enzyme solutions

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* See (Urnes and Doty, 1961).

containing 10 ug were applied in small spots to the thin layers and several amino acids in 1 ug quantities were similarly applied for comparison purposes. Trypsin and α-chymotrypsin solutions gave some faintly ninhydrin positive areas distributed between the origin and approximately 2/3 the distance to the solvent front. The irradiated samples and controls gave identical patterns and color intensities, indicating that only autolysis was responsible for the material detected. None of the other enzymes displayed any evidence of degradation.

B. Noncavitating Ultrasonic Irradiations of Enzyme Catalyzed Reactions

The following was the procedure for observing the effects of noncavitating ultrasound on enzyme catalyzed reactions using the specially constructed irradiation cell described in Section II, C, 1 and the Appendix. The electronic components were tuned-up with distilled water in the irradiation cell; the distilled water was then replaced with the degassed substrate for the reaction to be studied. After setting the absorbance at some arbitrarily selected position on the recorder chart (refer to Figure 9 a), the substrate was irradiated for one minute, in the absence of enzyme, to determine whether ultrasonic irradiation produced any change in absorbance, i.e., affected the substrate. The temperature was monitored during the irradiation by a thermistor probe (Tele-Thermometer, Yellow Springs Instrument Co.) inserted in the filling hole of the sample irradiation cell. When assured that irradiation had no effect upon the substrate (other than the effects due to light diffraction or temperature change which will be discussed later), the enzyme was added to start the reaction. After the reaction had proceeded one minute, the ultrasound was turned on for one minute while the spectrophotometrically monitored rate of reaction, $\Delta A/t$, was recorded continuously on the chart paper. After one minute of ultrasonic irradiation, the ultrasound was turned off and the reaction was allowed to continue as long as the reaction rate remained linear on the logarithmic recording. The temperature was monitored continuously and any changes were noted on the recorder chart.

Several interrelated factors had to be considered in the selection of the procedure outlined above, and the following remarks describe the instrumental limitations from which the procedure evolved. The rate of an enzyme catalyzed reaction depends on the concentrations of enzyme and substrate and

FIGURE 9

TYPICAL IRRADIATED ENZYME ASSAYS

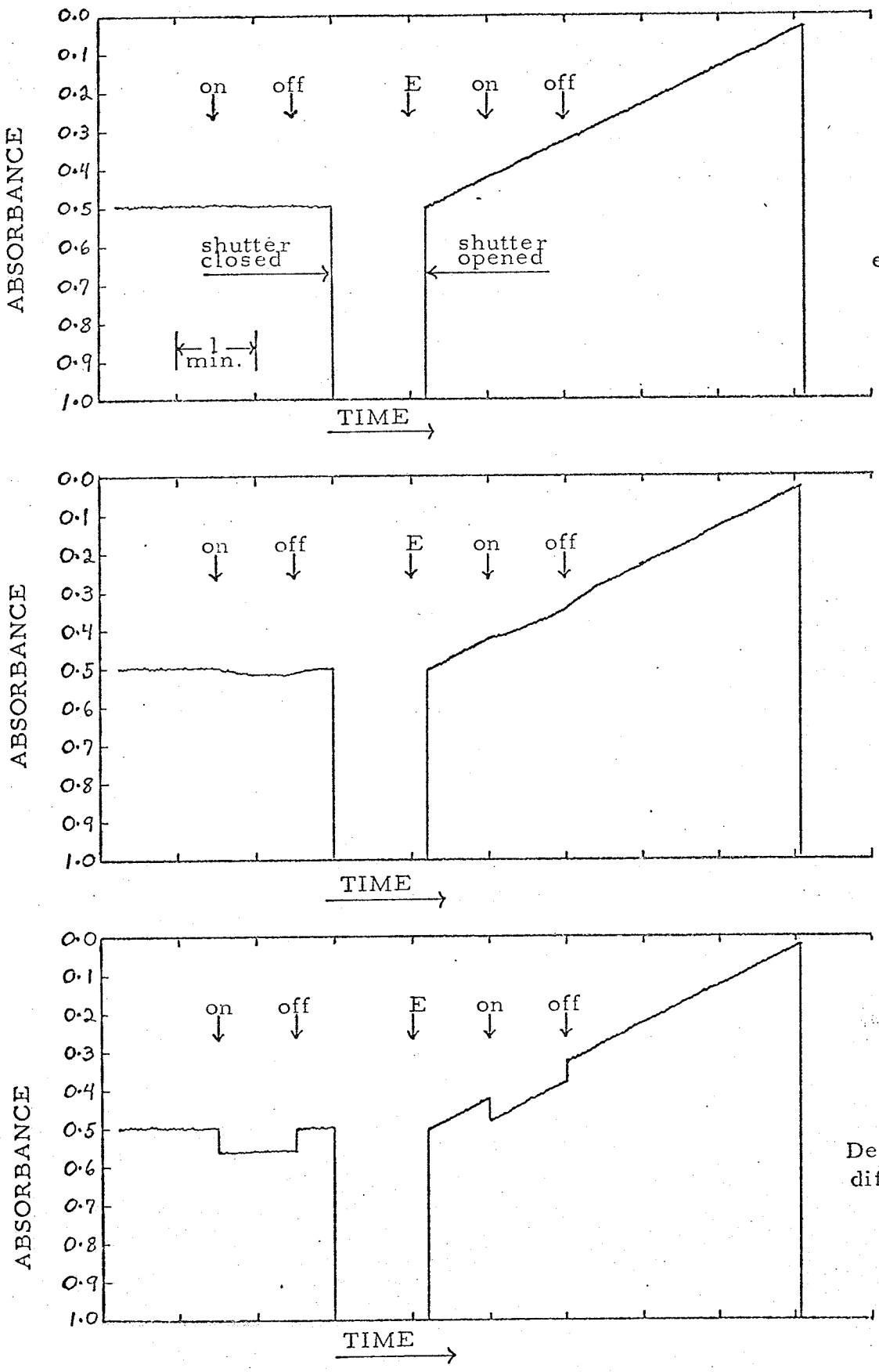
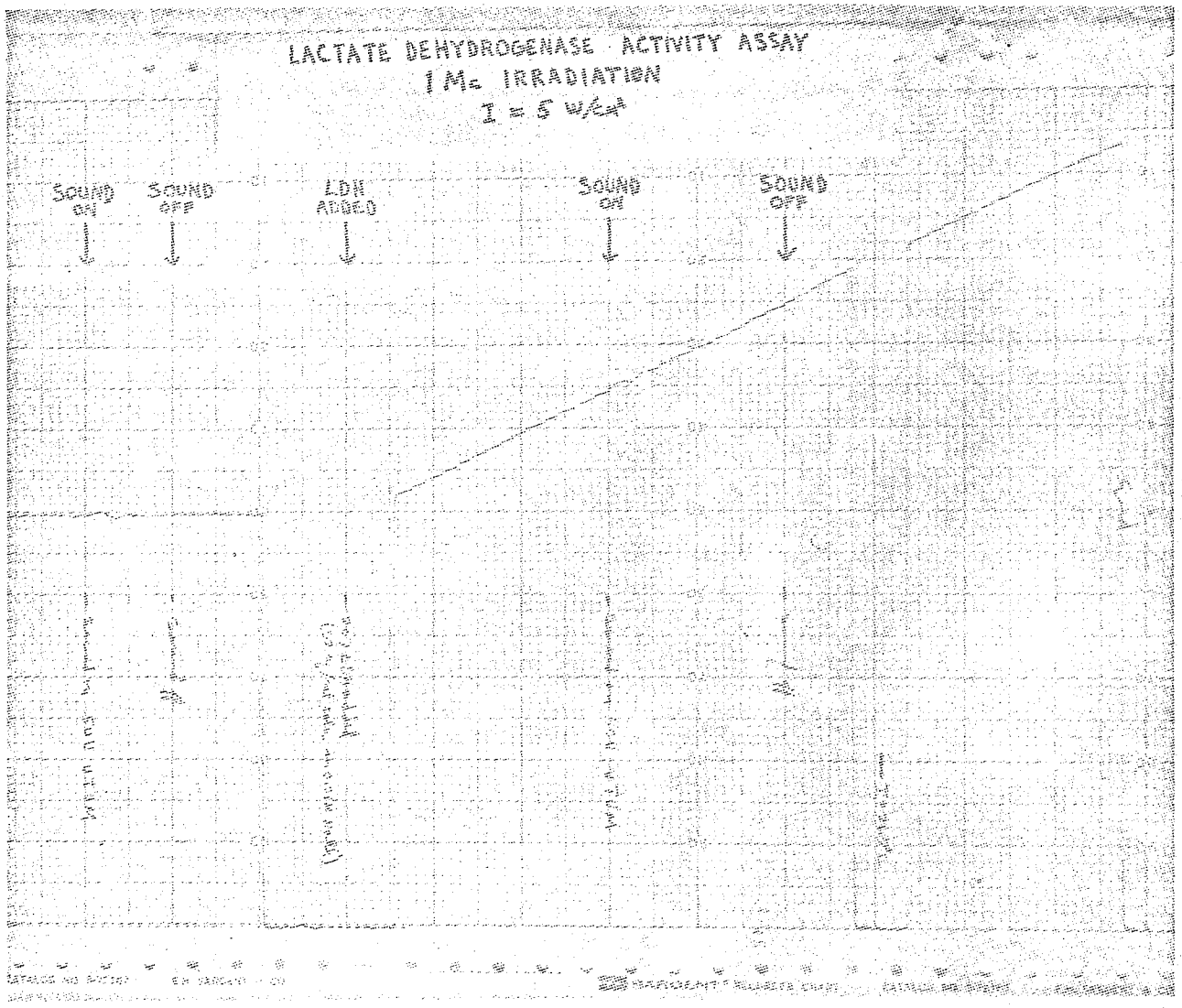


FIGURE 9 (continued)



(d.) actual data

(a.), (b.) and (c.) are drawings representing the types of effects observed when ultrasound interacts with substrate solutions and enzyme assay reactions. ON and OFF indicate the commencement and termination of the ultrasonic irradiation. E indicates when the enzyme was added to start the reaction. (a.) shows the sequence of events (described in the text) for an enzyme assay which was unaffected by ultrasonic irradiation. (b.) shows an assay in which $\Delta A/\Delta T$ is the only effect of ultrasonic irradiation. (c.) shows an assay in which Debye-Sears diffraction causes an apparent increase in absorbance. The effects illustrated by (b.) and (c.) are shown separately for the sake of clarity but can occur together in the same assay. (d.) is a photograph of actual data for an assay of the type drawn in (a.).

on the temperature and pH of the reaction mixture. Temperature and pH are held constant according to the conditions specified by the definitions for a unit of catalytic activity given in II, B. The sensitivity of the spectrophotometer limits the range of substrate concentrations which may be used, and within the useful range, preliminary experiments were carried out to determine the best combination of enzyme and substrate concentrations which yields a suitable first-order reaction for study. The criteria for suitability are a reaction whose rate is sufficient to enable detection of a change in rate of 5% or less, and a reaction that remains first order for at least three minutes. Three minutes is considered a minimum time for the reaction to remain first order because it is desirable to be able to observe the reaction for one minute both before and after a one-minute ultrasonic irradiation. Irradiations longer than one minute usually were undesirable at the intensities employed because they resulted in temperature increases which could not be brought back to the initial level before the reaction ceased to be first order. Since enzyme catalyzed reactions are temperature dependent, approximately doubling in rate for a $10^{o}C$ temperature increase, it was desirable not only to maintain constant temperature during a given enzyme assay but also to assay at the same temperature when comparing the activities of different samples. If, in addition, the enzyme catalyzed reaction is ultrasonically irradiated, it is necessary to monitor the temperature throughout the reaction because absorption of ultrasound by the reaction mixture produces a temperature rise. By limiting the irradiation times to one minute, intensities higher than 35 w/cm^2 could be employed and the reaction mixture in the thermostated irradiation cell could still be quickly restored to its initial temperature after irradiation.

Two phenomena which complicate the interpretation of the results are a substrate absorbance change with increased temperature and the Debye-Sears effect.

Temperature increases, due to acoustic absorption, changed the light absorbance ($\Delta A/\Delta T$) of solutions of ATEE, BAEE, cytidine 2':3' cyclic phosphate, and solutions of their reaction products. For the maximum temperature increases produced during irradiation, the observed ΔA (exaggerated in Figure 9 b) was approximately 5% of the total change observed in the assay. However, since the original temperature was quickly re-established after the acoustic exposure and since irradiation of substrate alone had previously provided knowledge of the time course of $\Delta A/\Delta T$, the effects of $\Delta A/\Delta T$ were readily separated from other ultrasonic effects on the reaction rate. It was particularly easy to detect the effect of $\Delta A/\Delta T$ in the case of the α-chymotrypsin assay since the assay produces a decrease of $A_{237\ m\mu}$ while $\Delta A/AT$ produces an increase.

The diffraction of light passing through a transparent medium which is also transmitting a beam of parallel ultrasonic waves is known as the Debye-Sears effect (Hueter and Bolt, 1955). Diffraction occurs as a result of the slight differences in refractive index between the alternate regions of compression and arefaction in the medium and, hence, the ultrasonic waves may be considered a three-dimensional diffraction grating traveling with the speed of sound. However, since the speed of light is much greater than the speed of sound, the diffraction grating is effectively stationary. In the resulting diffraction pattern, the light intensity of the zeroth order line is inversely proportional to the acoustic intensity; that is, the greater the acoustic intensity, the greater is the proportion of light diverted from the zeroth order to higher orders (Blitz, 1963). Debye-Sears diffraction affects

the form of the experimental results since the acoustic intensity is sufficient to diffract light into higher orders which do not reach the detector, the result on the recorder chart is an apparent increase in absorption (Figure 9c). The angle of diffraction, θ, when a parallel beam of monochromatic light of wavelength λ is orthogonally incident on an acoustic grating of progressive waves, is given by:

$$\sin \theta_n = n \lambda / d$$

where n is an integer representing the diffraction order and d is the acoustic wavelength. The circular window leading to the detector is 2 cm in diameter and is located 15 cm from the center of the reaction irradiation cell. Thus, the lowest order to fall completely outside the window must be displaced 1 cm to the left or right of the zeroth order line at a distance 15 cm from the acoustic grating. By solving the relation given above using θ = arctan (1/15) and the shortest light wavelength used for any of the assays (237×10^{-7} cm), the lowest order excluded from the detector was calculated for each of the ultrasonic frequencies employed.

Freq. (MHz)	d (cm)	Lowest Order Completely Excluded
1	0.15	422
9	1.67×10^{-2}	47
27	5.56×10^{-3}	16

Since the window is circular and the slit image completely crosses this circle, all the diffraction orders for $n > 0$ will be partially excluded from the detector and it is porbably this partial exclusion at orders much lower than the first one completely excluded that accounts for most of the apparent light absorption increase due to diffraction. The diffraction presents no

difficulty in the interpretation of data, however, because it merely represents an abrupt displacement of the absorbance to a higher level marking the commencement of ultrasonic irradiation. The reaction rate, $\Delta A/t$, continues to be recorded only displaced by a fixed amount (Figure 9 c). When the ultrasound was turned off, the absorbance abruptly decreases by this same amount while the reaction continues.

Care must be taken in adding the enzyme to the degassed substrate so that air bubbles are not introduced into the reaction mixture. The most satisfactory method was to deliver the enzyme from a lambda pipette at the surface of the substrate and then carefully stir the reaction mixture with the pipette. Delivery of the enzyme, stirring of the reaction mixture, and positioning the thermistor probe in the filling hole of the reaction irradiation cell could all be accomplished in 20 sec. or less.

Degassing the substrate for the 1 MHz irradiations was a precaution taken to insure the absence of cavitation, although the intensity employed at 1 MHz was below the cavitation threshold for aerated water (see Figure 1). If cavitation had occurred in the reaction mixture, it would have been detected because the cavitation bubbles scatter light causing an erratic increase of absorbance.

Table 8 gives the amount of enzyme, the substrate concentration, and the pH in the ultrasonically irradiated reaction mixture.

The results of the ultrasonic irradiations of the enzyme catalyzed reactions are listed, according to the frequency and intensity employed, in Table 9 . All the irradiations were continuous for one minute and the sample irradiation cell was maintained, except for the brief temperature rise indicated, at $25.0 \pm 0.1^{\circ}C$. 1 MHz ultrasound and the lowest intensity

TABLE 8

CONDITIONS OF THE IRRADIATED REACTIONS

Enzyme	μg Enzyme Assayed	Substrate Conc.*	pH
α-Chymotrypsin	10	2.89×10^{-4} M ATEE	7.0
Trypsin	10	7.22×10^{-5} M BAEE	7.0
Lactate Dehydrogenase	0.62	3.45×10^{-4} M Pyruvate 6.90×10^{-5} M $NADH_2$	7.4
Aldolase	10	4×10^{-3} M Fructose-1, 6-diphosphate 2.3×10^{-3} M Hydrazine Sulfate	7.3
Ribonuclease	100	0.1 mg/ml cytidine 2':3' phosphate (4.68×10^{-4} M)	7.1

* See Section II, B for buffers used and assay details.

TABLE 9

EFFECTS OF INTENSE NONCAVITATING ULTRASOUND ON ENZYME CATALYZED REACTIONS

Enzyme of the Irradiated Reaction	Frequency (MHz)	Intensities (w/cm^2)	Max. Temp. Rise During Irradiation (^{o}C)	% Native Activity
α-Chymotrypsin	1	5	0.5	100
	9	0.5, 3, 23	<0.1, 0.5, 2.5	100, 100, 100
	27	1	1.0	100
Trypsin	1	5	0.5	100
	9	0.5, 4, 23	<0.1, 0.7, 3.3	100, 100, 100
	27	1	1.0	100
Lactate Dehydrogenase	1	5	0.5	100
	9	0.5, 14, 39	<0.1, 0.7, 1.5	100, 100, 100
	27	1	1.2	100
Aldolase	1	5	0.5	100
	9	0.5, 12, 37	<0.1, 0.8, 1.5	100, 100, 100
	27	1	1.2	100
Ribonuclease	9	14, 39	0.8, 1.7	100, 100
	27	1	1.0	100

1 Minute (continuous) Irradiations at 25°C

(0.5 w/cm^2) at 9 MHz (for the first four enzymes of Table 9) were generated by a crystal with a 1 MHz fundamental resonant frequency. The higher intensities at 9 MHz and 27 MHz were generated by a crystal with a 9 MHz fundamental resonant frequency.

It is seen that none of these irradiations (Table 9) had any effect on the enzyme catalyzed reactions.

C. Effects of Cavitation on Enzymes in Solution

The objective of the experiments reported here was to verify that cavitation is a necessary condition for the denaturation of enzymes by ultrasound, and to report the effects of cavitation on two enzymes not previously investigated. Since the results in Sections A and B show that intense noncavitating ultrasound has no effect on the enzymes in solution, if it can be established that cavitation denatures the enzymes, then it can be concluded that cavitation is a necessary condition for ultrasonic denaturation of the enzymes in solution.

The first thorough study of the effects of cavitation on a purified enzyme in solution was the report on pepsin by Chambers (1937). Subsequently, other reports on enzymes of varying degrees of purity have appeared in the technical literature and recently they were reviewed by El'piner (1964c). The published reports represent investigations employing a wide variety of equipment, techniques and degrees of biochemical sophictication. Since the number of these investigations is still relatively small, it is not surprising that a variety of techniques and equipment are in use. The prevailing attitude implicit in most studies seems to be that the intensity and frequency employed are relatively unimportant as long as cavitation is produced. The most common features of these studies are enzyme inactivation and dependence of the activity loss on the gas present during the irradiation. Usually the presence of oxygen (air) leads to the greatest loss of enzyme activity, while irradiations carried out in solution saturated with hydrogen show little or no activity loss although changes in the macromolecular structure may be detectable.

Of the five enzymes whose interactions with noncavitating ultrasound are

reported in A and B only trypsin and ribonuclease have been analyzed after treatment with cavitating ultrasound. Most investigators have been content to demonstrate enzyme inactivation after cavitation; however, these studies of trypsin and ribonuclease include several types of analysis on the cavitated samples.

El'piner et al. (1959) observed a decrease in both enzymatic activity and molecular weight (as measured by the surface pressure technique) when air-saturated trypsin solutions were cavitated, but cavitation of hydrogen-saturated trypsin solutions increased the molecular weight 30 per cent without any loss of enzymatic activity. Polarographic analyses (Bronskaya and El'piner, 1963) revealed that the double protein wave typical of native trypsin disappeared after cavitation in the presence of oxygen, but remained after cavitation of hydrogen-saturated trypsin solutions. The U.V. absorption spectra of the cavitated samples were also measured. The samples saturated with hydrogen before cavitation gave U.V. spectra identical to native trypsin while samples cavitated in the presence of oxygen displayed a general overall absorbance increase whose magnitude depended on the duration of the irradiation. Since the absorbance increase of these samples was less in the region 275 mμ to 285 mμ than at the shorter wavelengths, El'piner concluded that the aromatic side chains of tyrosine and tryptophan residues have been destroyed (El'piner et al., 1959). This interpretation is a possible one; however, the greater absorbance increase at shorter wavelengths is usually indicative of Rayleigh scattering due to aggregate formation. In the absence of more direct evidence for the destruction of aromatic amino acid residues, to interpret the relatively small absorbance increase at 280 mμ as a decrease seems an unwarranted abuse of the data.

El'piner and Zorina (1960) studied the effects of cavitation on ribonuclease. They observed large increases in the U.V. absorption spectrum, increase in molecular weight due to aggregation, but no loss of enzymatic activity. All these effects were observed regardless of whether the gas present in the cavitated sample was hydrogen or oxygen; however, the effects were more pronounced when oxygen was present. Cleavage of low molecular weight fragments from ribonuclease molecules was inferred from the results of dialysis experiments on the irradiated samples. After dialysis the U.V. spectrum of an irradiated sample became more nearly like the native spectrum and this fact was interpreted to mean that low molecular weight material (presumed to be responsible for the great increase in the U.V. absorption spectrum) had been removed by dialysis. However, since no activity loss was reported and aggregation was observed, a simpler explanation which would eliminate the hypothetical fragments is that dialysis promoted dissociation of light scattering aggregates.

The experiments reported below are not intended to be comprehensive studies and no particular effort was made to duplicate the conditions of other investigators. Indeed, the latter would be a futile objective since most equipment was not commercially available and often key information concerning techniques was not included in the literature reports.

The three enzymes selected for investigation of cavitation effects were trypsin, α-chymotrypsin and lactate dehydrogenase (LDH). Trypsin was selected to determine if the equipment employed would give results comparable to those of El'piner et al. (1959) discussed above. LDH and α-chymotrypsin were chosen because they have not been studied previously.

All experiments were carried out in air (i.e., the presence of oxygen)

according to the procedures described in II, C, 3. Control samples were treated exactly as the irradiated samples except for the irradiation. The solutions cavitated with the 20 KHz equipment are as follows:

Enzyme	Conc. (mg/ml)	Solvent	pH
Trypsin	0.92	tris buffer	7.1
α-Chymotrypsin	10.20	tris buffer	7.1
	0.95	tris buffer	7.1
LDH	6.20	17% sat. $(NH_4)_2SO_4$	5.8
	0.62	1.7% sat. $(NH_4)_2SO_4$	6.5

The effects of the 20 KHz cavitation on enzyme activity are given in Table 10 and enzyme activity loss as a function of cavitating irradiation time is shown for the 0.95 mg/ml α-chymotrypsin solution and the 0.62 LDH solution in Figures 10 and 11, respectively.

The 5-min. cavitated sample and the control for the 0.95 mg/ml α-chymotrypsin solution were also assayed at substrate concentrations one-half and twice the concentration specified in II, B. The results of the assays performed on 2 μg of control and the 5-min. cavitated sample are given below, where S represents substrate (ATEE) concentration and v_o and v_5 are the reaction velocities for reactions catalyzed by the control and 5-min. cavitated sample, respectively. These results are plotted in the form

S(M)	$\frac{1}{S}$ (M^{-1})	v_o ($\Delta A_{237\ m\mu}$/min.)	$\frac{1}{v_o}$	v_5 ($\Delta A_{237\ m\mu}$/min.)	$\frac{1}{v_5}$
.0005	2000	.013	77	.009	111
.0010	1000	.017	59	.012	83
.0020	500	.020	50	.014	72

TABLE 10

EFFECTS OF 20 KHz ULTRASONIC CAVITATION ON ENZYME ACTIVITY

Enzyme	Conc. (mg/ml)	Irradiation Time (min.)	Temp. Range (^{o}C) During Irradiation	% Control Activity
Trypsin	0.92	30	1.5 to 19.0	69
α-chymotrypsin	10.20	1	2.0 to 13.0	100
	10.20	5	2.0 to 13.0	100
	10.20	10	2.0 to 13.0	100
	0.95	1	2.0 to 14.0	94
	0.95	5	2.0 to 18.5	71
	0.95	15	2.0 to 18.0	69
LDH	6.20	15	2.0 to 19.0	37
	0.62	1	2.0 to 14.0	89
	0.62	5	2.0 to 17.0	61
	0.62	15	2.0 to 18.0	25
	0.62	21	2.0 to 18.0	11
	0.62	30	2.0 to 17.5	4

FIGURE 10

EFFECT OF CAVITATION ON α-CHYMOTRYPSIN ACTIVITY

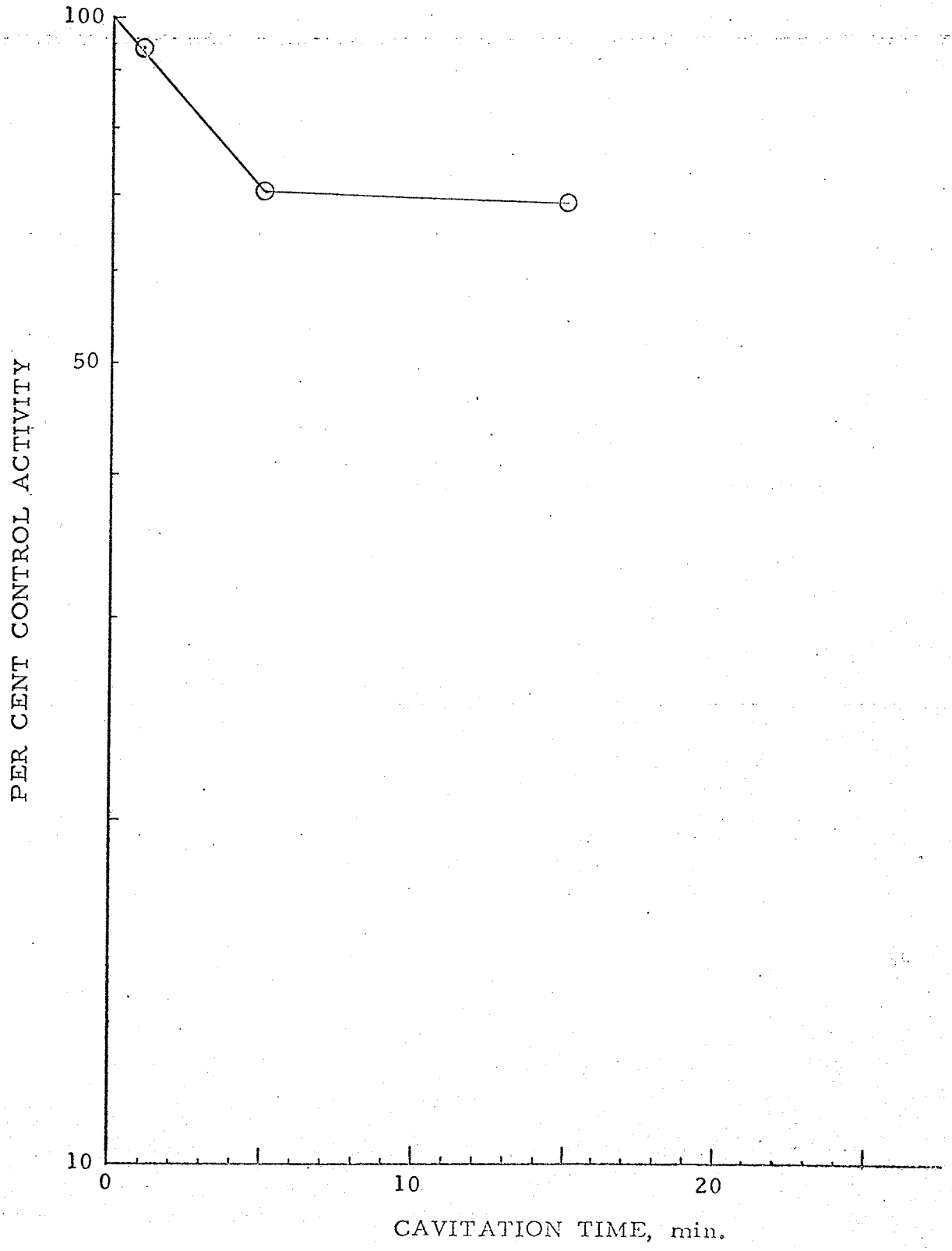


FIGURE 11

EFFECT OF CAVITATION ON LDH ACTIVITY

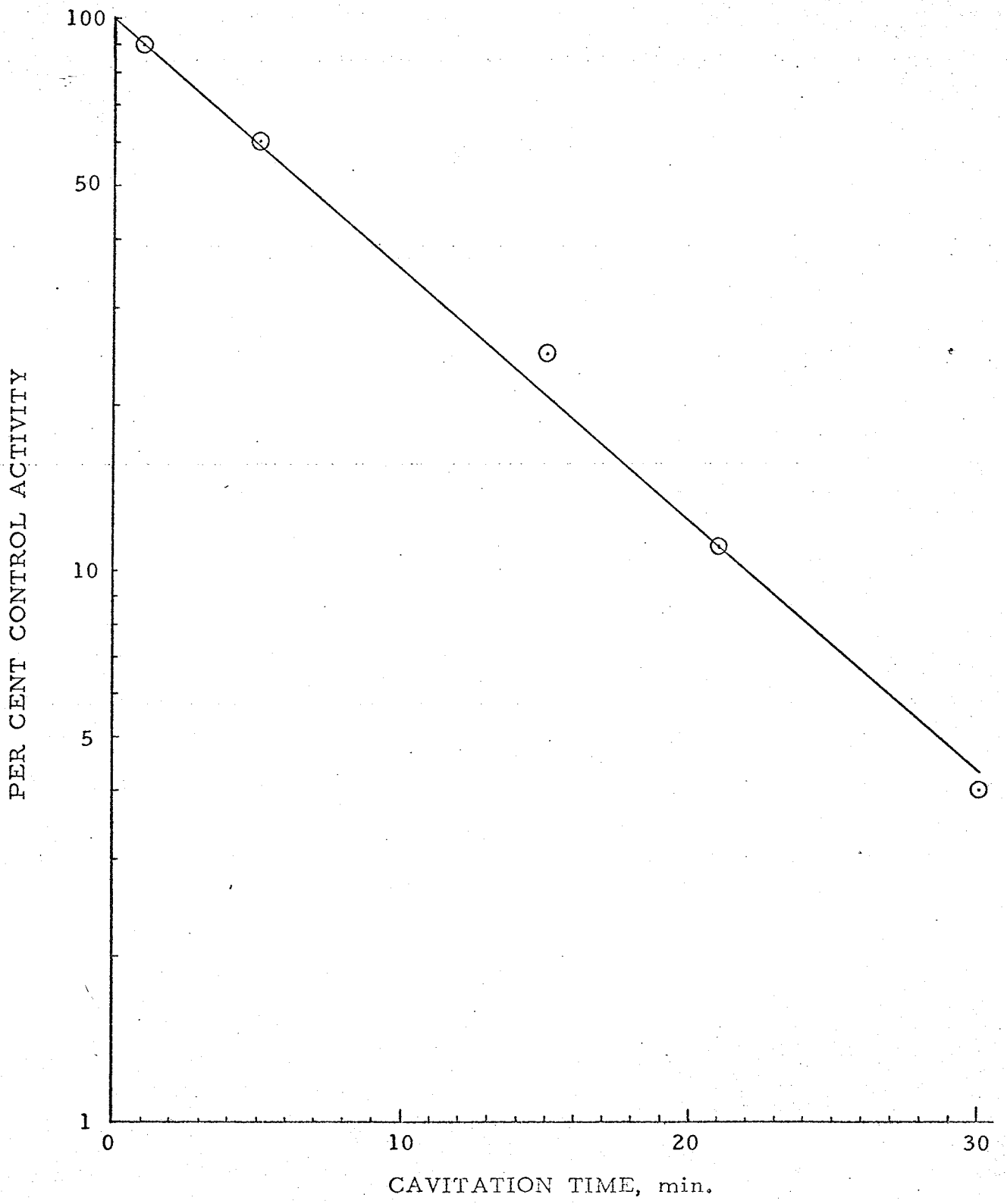


TABLE 11

U.V. ABSORPTION MAXIMA AND MINIMA

Enzyme Sample (dil.)	$A_{280\ m\mu}$	$A_{250\ m\mu}$	$A_{280\ m\mu}/A_{250\ m\mu}$
Trypsin (0.92 mg/ml)			
Control (1:3)	0.479	0.184	2.61
30 min. Sample (1:3)	0.438	0.176	2.49
α-Chymotrypsin (10.20 mg/ml)			
Control (1:20)	0.879	0.315	2.79
10 min. Sample (1:20)	0.882	0.318	2.77
α-Chymotrypsin (0.95 mg/ml)			
Control (1:2)	0.878	0.307	2.86
1 min. Sample (1:2)	0.866	0.323	2.68
5 min. Sample (1:2)	0.848	0.328	2.59
15 min. Sample (1:2)	0.813	0.325	2.50
LDH (6.20 mg/ml)			
Control (1:20)	0.491	0.181	2.72
15 min. Sample (1:20)	0.356	0.134	2.66
LDH (0.62 mg/ml)			
Control (1:1)	0.906	0.334	2.71
1 min. Sample (1:1)	0.880	0.327	2.69
5 min. Sample (1:1)	0.830	0.309	2.69
15 min. Sample (1:1)	0.684	0.261	2.62
21 min. Sample (1:1)	0.665	0.269	2.47
30 min. Sample (1:1)	0.641	0.267	2.40

$\frac{1}{v}$ versus $\frac{1}{S}$ (Figure 12) so that the kinetic constants, K_m and V, of the Michaelis-Menten equation,

$$\frac{1}{v} = \frac{K_m}{V} \cdot \frac{1}{S} + \frac{1}{V} \qquad (20)$$

can be determined from the intercepts on the axes (Lineweaver and Burke, 1934). V in Equation (20) represents the maximum reaction velocity and the Michaelis constant $K_m = (k_2 + k_3)/k_1$ where k_1, k_2 and k_3 are rate constants in the process of complex formation between enzyme (E) and substrate (S) and the subsequent breakdown of the complex (ES) into the products (P) and free enzyme:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P + E$$

For ATEE, K_m approximates a true dissociation constant for ES (Cunningham and Brown, 1956) and, thus, it provides a measure of enzyme-substrate affinity which is useful in assessing damage to the enzyme molecules.

Comparison of the U.V. absorption spectra of controls to those of the cavitated samples is made by listing (see Table 11) the absorption maxima at 280 mu and minima at 250 mu and the ratios of the maxima to the minima.

The specific rotation and sedimentation coefficient were determined for the control and 15-min. cavitated sample of the 6.20 mg/ml LDH solution. The results are given in Table 12.

TABLE 12

PROPERTIES OF CAVITATED 6.20 mg/ml LDH

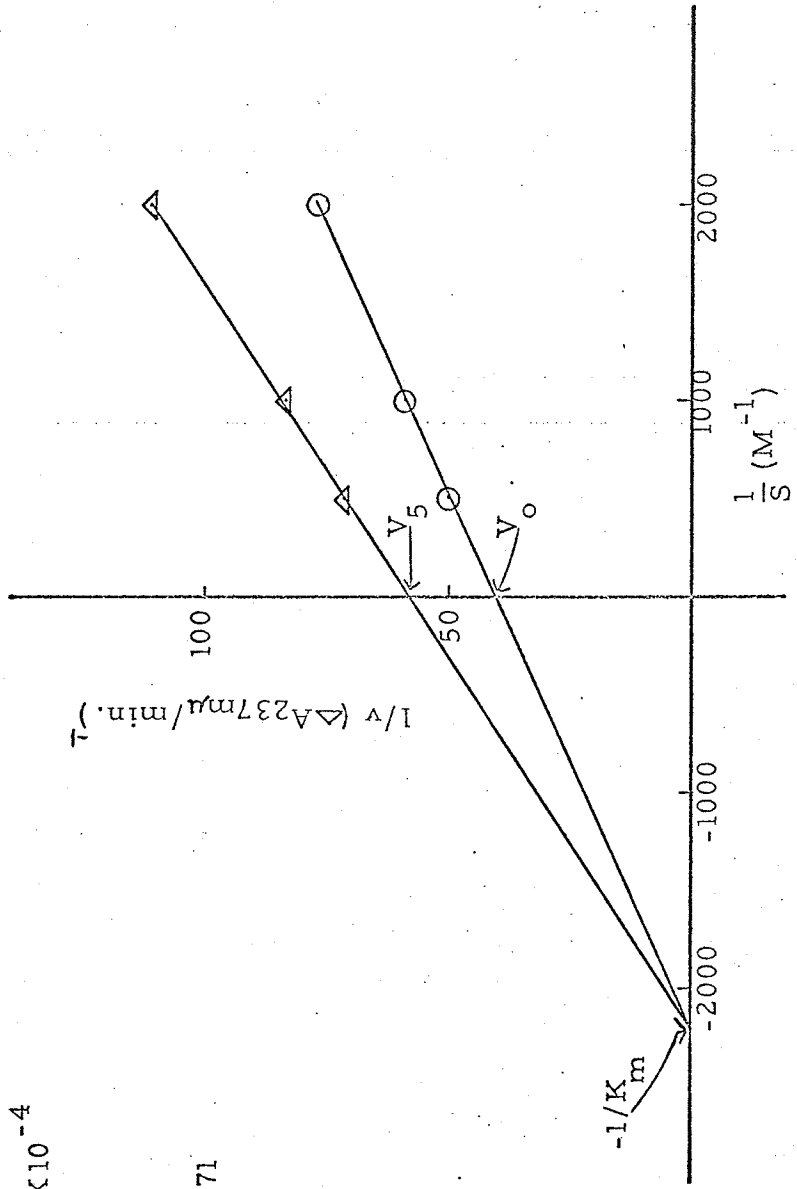
	$[\alpha]_D$	$S_{20,w}$
Control	-38^o	5.78, (5.54)*
15-min. sample	-50^o	3.94, (5.52)*

* minor peak

FIGURE 12

LINEWEAVER-BURK PLOT

FOR CONTROL AND 5 MINUTE CAVITATED SAMPLE



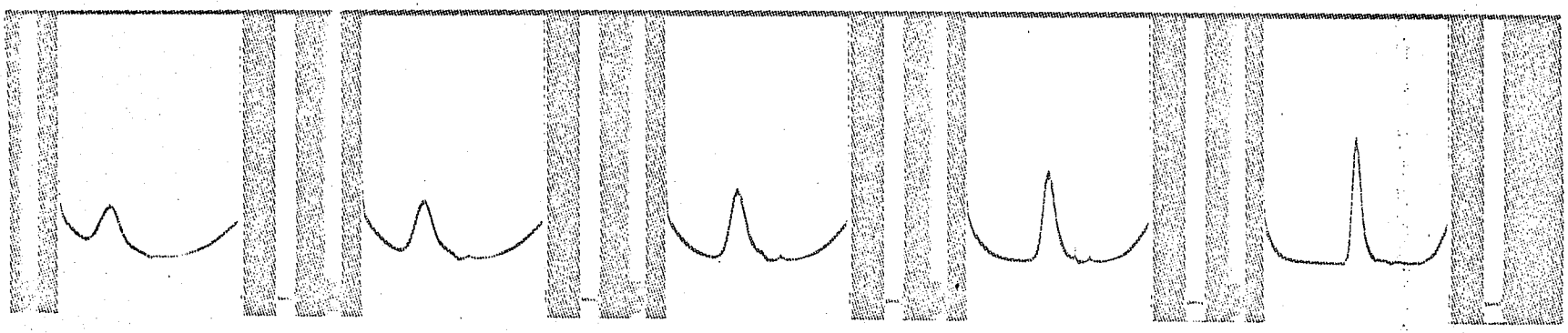
The schlieren patterns are shown in Figure 13.

Precipitation of denatured protein was observed in all the cavitated samples of Table 10 which showed activity loss. The U.V. spectra of Table 11 were read on the samples after removing the precipitate by centrifugation or filtration. The post-cavitation turbidity in the 0.92 mg/ml trypsin and 0.95 mg/ml α-chymotrypsin samples was slight and difficult to detect by eye. Centrifugation of the samples revealed small amounts of a cloudy gel-like precipitate and the more extensively degraded samples yielded more of the precipitate. The flocculent precipitate in the LDH samples was removed by filtration. The data of Table 11 reflect the protein loss due to precipitation since the 280 mμ maximum which is directly proportional to the protein concentration decreases with the activity of the sample. However, the absorbance at 280 mμ does not decrease to the same extent that the activity does, indicating that the precipitate-free cavitated samples contain inactive enzyme, i.e., not all the inactive enzyme precipitates. Furthermore, without accurate measurements of the amount of precipitated protein it is impossible to know if the observed U.V. absorption spectrum is different from the native spectrum at the same concentration. For example, it is impossible to attribute the magnitude of the absorption maximum to an increase or decrease due to soluble but damaged molecules without knowing the concentration, and it is also impossible to attribute the maximum to a certain concentration because the soluble but inactive molecules may have altered absorptivities. The decrease in $A_{280\ m\mu}/A_{250\ m\mu}$ with decrease in activity of the samples suggests that the soluble but inactive enzyme does alter the U.V. absorption spectrum.

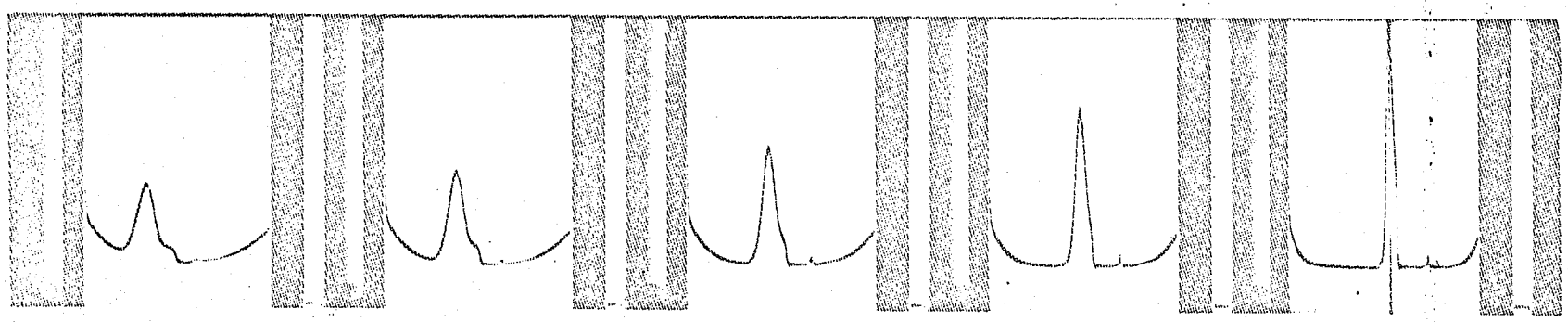
The cavitation of 0.92 mg/ml trypsin in air for 30 min. resulted in an activity loss of 31 per cent. El'piner et al. (1959) observed an activity

FIGURE 13

LDH SEDIMENTATION VELOCITY PATTERNS



15 MINUTE CAVITATED SAMPLE, TABLE 12



CONTROL, TABLE 12

loss of 85 per cent after cavitating 0.20 mg/ml trypsin in air for 30 min. The differences in equipment, procedure, concentration, pH and method of activity assay make more than a qualitative comparison difficult. However, the fact that α-chymotrypsin (an enzyme very similar to trypsin) appears to be more susceptible to cavitation denaturation as the concentration decreases (see Table 10) may mean that this same factor, lower concentration, is principally responsible for the greater inactivation of trypsin observed by the Russian workers. El'piner et al. (1959) did not report precipitation of denatured trypsin. However, since precipitation was only barely visible at a concentration of 0.92 mg/ml in the experiment of Table 10, at concentrations only about one-fifth this amount turbidity may have existed and not been detected by eye. If the trypsin solutions of El'piner et al. (1959) were slightly turbid, the reported overall U.V. absorption increase whose magnitude depended on cavitation time would be explained.

Figure 11 shows that the rate of activity loss, $\frac{dA}{dt}$, of the 0.62 mg/ml LDH solution exposed to cavitation is directly proportional to the remaining activity, A, at any time, t:

$$\frac{dA}{dt} = - k A \qquad (21)$$

Integration of this equation gives $A = A_o e^{-kt}$ where A_o is the initial activity of the solution. Calculation reveals that the constant proportionality for Figure 11 is k = 0.105. Chambers (1937) reported exponential loss of activity for cavitated pepsin solutions, but, unfortunately, neither his data nor the curve drawn through the data are exponential. None of the explanations for the effects of cavitation mentioned in Section I can be either proved or eliminated on the basis of the observed inactivation kinetics,

although similar inactivation kinetics for enzyme solutions irradiated with X-rays suggest that perhaps the cause of enzyme inactivation in the case of cavitation is the same as for ionizing radiation, viz., the chemical effects of free radicals.

LDH was also cavitated for 15 min. at a concentration of 6.20 mg/ml and activity loss, precipitation of denatured enzyme and decrease in the U.V. absorption spectrum resulted (see Tables 10 and 11). After removing the precipitate, the specific rotation and the sedimentation coefficient of the cavitated sample and control were determined (see Table 12). The increase in levorotation indicates the presence of some denatured enzyme in the filtrate of the irradiated sample, although it must be remembered that the $[\alpha]_D$ was calculated using a spectrophotometrically determined concentration for the filtrate and, if the filtrate contains molecules with altered absorptivities, then the concentration and, hence, the calculated $[\alpha]_D$ may be erroneous. The sedimentation coefficient of the cavitated LDH is considerably reduced indicating either fragmentation to smaller molecules or an unfolding which increases the frictional coefficient of the molecules. Both schlieren patterns show a major peak, a minor peak of about 5.5S and a shoulder which separates from the major peak during sedimentation. Since the 15-min. cavitated sample possessed 37 per cent native activity and no peak appears in its schlieren pattern with a native S value, it seems likely that the major peak in the cavitated sample schlieren pattern represents intact but unfolded molecules with 37 per cent native LDH activity. The alternative conclusion is that the major peak represents a nearly 100 per cent conversion of 5.78S molecules into 3.94S fragments and that these fragments possess 37 per cent of the activity of the native molecules.

Alpha-chymotrypsin at a concentration of 10.20 mg/ml is completely resistant to the cavitation employed in these experiments (see Tables 10 and 11). The inactivation of the 0.95 mg/ml α-chymotrypsin solution follows Equation (21) for 5 min. and then becomes more resistant to inactivation (Figure 10). This result might be interpreted to mean that after 5 min., the enzyme was degraded as far as was possible under these irradiation conditions and that the solution contained enzyme molecules each with 70 per cent of its original activity. This possibility seems to be ruled out by the results shown in Figure 12, for if the enzyme molecules are damaged but partially active it is expected that their affinity for substrate molecules will be reduced and, hence, that their K_m will be greater than the K_m of the native enzyme. Figure 12 shows that the 5-min. cavitated α-chymotrypsin has the native K_m but a reduced maximum velocity, indicating that fully active enzyme is present only in reduced amount.

Thin layer chromatography was done on the 15-min. cavitated 6.20 mg/ml LDH solution, the 30-min. cavitated 0.95 mg/ml α-chymotrypsin solution, the 30-min. cavitated 0.92 mg/ml trypsin solution and appropriate controls. Five micrograms of each sample were analyzed as described in II, D, 5. No amino acids or small peptides were detected in any of the samples.

A final experiment was performed to verify that cavitation damage could be produced in the intense focussed systems used for the noncavitating ultrasound studies. Using the horizontal focussed system (II, C, 2) at 1 MHz, an aerated 6.5 ml, 1 mg/ml, pH 3, α-chymotrypsin solution was cavitated for 18 min. The cavitation produced was not nearly as vigorous as in the 20 KHz system. The temperature of the medium was 27.3°C throughout the cavitation period. The sample showed 91 per cent control activity. Thus, cavitation

can be produced, though with difficulty, in the horizontal focussed system and, in view of the results of Section A, cavitation appears to be necessary in order to denature enzymes in solution.

IV. CONCLUSIONS

The results of this study (Chapter III) lead to the following conclusions. Irradiation of ~ 1 per cent solutions of α-chymotrypsin, trypsin, lactate dehydrogenase, aldolase and ribonuclease with noncavitating ultrasound at dose levels sufficient to cause extensive structural and functional damage in tissues, has no effect on either the structure or function of the enzymes. That the catalytic function of the five enzymes was unaffected by intense noncavitating ultrasound was demonstrated in two ways: the ~ 1 per cent solutions showed full catalytic ability when assayed after irradiation (Section III,A), and irradiations failed to inhibit the enzymes while they were in the process of catalyzing a reaction (Section III,B). Structural integrity of the irradiated enzyme samples was demonstrated by the lack of any significant differences between the U.V. absorption spectra, specific rotations, sedimentation coefficients and thin-layer chromatographic analyses of the irradiated samples and unirradiated controls (Section III,A). In addition, experiments with trypsin, α-chymotrypsin and lactate dehydrogenase showed that cavitating ultrasound denatured these enzymes such that enzymatic activity was lost and, in the case of lactate dehydrogenase, physico-chemical measurements revealed changes in molecular structure as well. It is concluded that cavitation is a necessary condition for damage to be produced by ultrasound in enzyme solutions *in vitro*.

These conclusions raise several points worthy of further discussion. First, the inability of intense noncavitating ultrasound to damage the enzymes, particularly trypsin solutions, contradicts the findings of Stefanović *et al.*, (1958-59). These investigators irradiated 10 ml samples of 2.5 mg/ml solutions of trypsin in distilled water with 3 MHz noncavitating

ultrasound at an intensity of 2.5 w/cm^2. The samples were contained in glass cylinders closed at one end by a thin rubber membrane and the cylinder, closed end down, was partially immersed in distilled water that covered the transducer. Irradiation was upward, the ultrasound being transmitted to the sample through the distilled water and the thin rubber membrane. Care was taken to keep the temperature of the samples below 37^{o}C. These irradiations resulted in 6, 13, 27 and 31 per cent activity losses for trypsin solutions irradiated 10, 20, 30 and 45 min., respectively. Another report from the same laboratory (Stefanović et al., 1960) showed 14, 29, 43, 51 and 73 per cent activity loss for 5 mg/ml solutions of α-amylase (diastase) irradiated for 5, 10, 15, 20 and 30 min., respectively, according to the same procedure used for trypsin solutions which was described above.

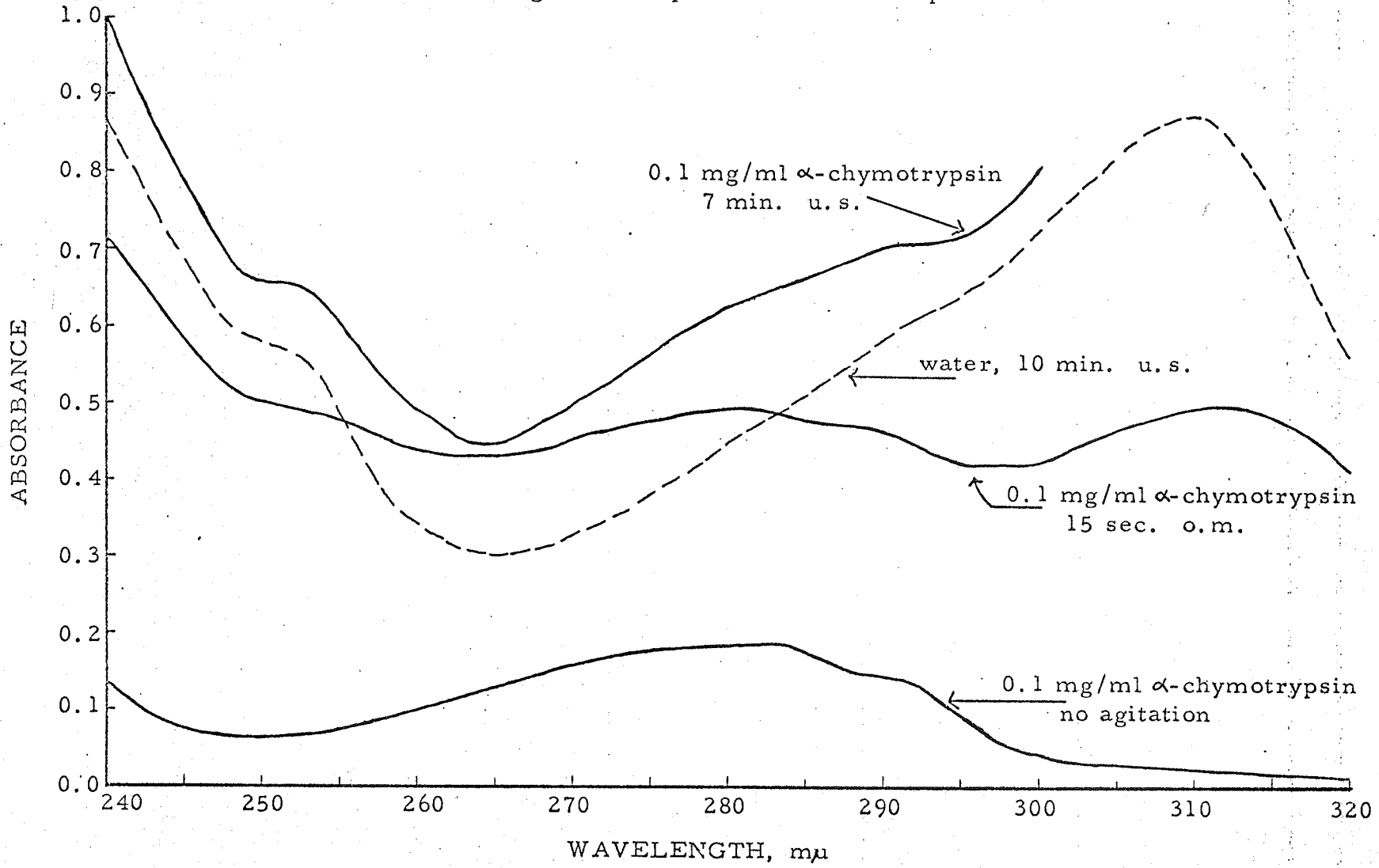
To resolve the discrepancy between the results of the experiments just described and the results of this report, experiments were performed which show that the enzyme inactivation observed by the Yugoslav investigators was due to the presence of the rubber membrane used to close their sample containers (Macleod and Dunn, 1966). A summary of these experiments follows.

Solutions of α-chymotrypsin at various pH's and concentrations were placed in small rubber bags and irradiated in the horizontal irradiation system (Section II,C,2) with 1 MHz noncavitating ultrasound at an intensity of 75 w/cm^2. Enzyme inactivations similar to those reported by Stefanović et al. (1958-59) for trypsin were observed. For example, 1, 3 and 6 min. irradiations of 1 mg/ml, pH 7, α-chymotrypsin solutions yielded activity losses of 20, 72 and 86 per cent, respectively. The U.V. absorption spectra of irradiated samples showed more than a 200 per cent increase over the entire wavelength range 240 to 300 mμ (Figure 14). Control samples in rubber bags

FIGURE 14

U. V. SPECTRA OF SOLUTIONS MECHANICALLY AGITATED IN THE PRESENCE OF RUBBER

u. s.: 1MHz ultrasonic irradiation, 75w/cm^2, in rubber bag
o. m.: omni-mixer agitation in presence of rubber pieces



which were not irradiated showed no changes either in enzymatic activities of U.V. absorption spectra. Figure 14 also shows the U.V. absorption spectra of two other solutions; one solution was distilled water which had been placed in a rubber bag and irradiated with 1 MHz noncavitating ultrasound at 75 w/cm^2 for 10 min. and the other was an α-chymotrypsin solution which was agitated in an Omni-mixer (Sorval) for 15 sec. in the presence of small pieces of a rubber bag. The latter solution showed a 64 per cent activity loss while a control sample also agitated for 15 sec. but in the absence of the rubber remained fully active. The irradiation of distilled water in a rubber bag shows that neither the enzyme nor any component of the buffer used is responsible for the presence of the strongly absorbing material in the solution and that this material undoubtedly comes from the rubber.

Two conclusions are drawn from these experiments. The inactivation of the enzyme solution in contact with rubber requires both the presence of the rubber and mechanical agitation, but the mechanical agitation need not be produced by ultrasound. Also, the mechanical agitation of an aqueous solution in contact with rubber causes the release of a material from the rubber into the solution which absorbs strongly in the U.V. and is able to inactivate enzymes. It is also concluded, on the basis of the experiments just described and the absence of any inactivation of the trypsin solutions in inert containers by ultrasound of much higher intensity reported in Section III,A, that the inactivation of trypsin reported by Stefanović et al. (1958-59) was not due to the direct effects of noncavitating ultrasound but to an inhibitor released by ultrasonic agitation of the rubber membrane used to close their sample containers.

Another interesting aspect of the results obtained with intense noncavitating ultrasound is the fact that, despite the expected absorption of

acoustic energy being many times greater than the energy required to denature the enzymes, the mechanisms of absorption and dissipation of this energy apparently do not involve permanent changes in the enzyme molecules nor detectable transient changes which in any way affect the normal functioning of the enzyme *in vitro*. The results suggest that the mechanism of acoustic energy absorption does not involve great changes in the secondary and tertiary structure of the enzyme molecules and this information may be useful to investigators studying the mechanisms of acoustic absorption by polymers in solution.

The results of this report also indirectly strengthen the belief that relative motion between molecules of DNA and solvent was responsible for degradation of DNA by intense noncavitating ultrasound (Hawley *et al.*, 1963). A calculation made in Chapter I showed that relative motion between the enzyme molecules and the solvent would not result in tensile forces large enough to break covalent bonds. The same calculation using the molecular dimensions for DNA reveals that relative motion can provide a tensile force sufficient to break covalent bonds of the helical backbone of DNA molecules. Thus, the fact that intense noncavitating ultrasound has no effect on enzymes in solution suggests that relative motion may be the only way intense noncavitating ultrasound can affect polymers in solution, and that molecules too small to develop appreciable tensile forces due to relative motion will not be affected. Further research is needed to establish the importance of relative motion as a mechanism for polymer degradation by intense noncavitating ultrasound.

The molecular biological approach in studying the interactions of intense noncavitating ultrasound with biological structures has revealed that DNA can

be degraded *in vitro*, but enzymes cannot. Consideration should now be given to interpretations of these facts in terms of the interactions of tissue with intense noncavitating ultrasound and to suggestions of promising approaches for future research.

Degradation of DNA *in vivo* cannot account for the effects observed in irradiated tissues since it is unlikely that the loss of cellular control processes involving DNA would result in the rapid functional changes observed. For example, irradiation of appropriate portions of the mammalian central nervous system can produce motor paralysis which is observable almost instantaneously (Fry, 1958). The inactivation of enzymes would provide a more reasonable explanation for the rapidly appearing effects in tissues since they are more directly concerned in the chemical reactions essential to the cells' survival. The fact that intense noncavitating ultrasound has no effect on enzymes *in vitro* does not necessarily imply that there are no interactions in the cellular environment. Some enzymes, such as the enzymes of the Krebs cycle which are located in mitochondria, apparently are structurally organized in groups. In the case of the Krebs cycle, the enzymes are closely associated with, and possibly bound to, the cristae of the mitochondria (Lehninger, 1960). Thus, it is possible that in this structured environment, an enzyme may be more susceptible to denaturation by intense noncavitating ultrasound either directly or by some indirect mechanism involving the adjacent structures. Use of histochemical techniques such as those employed by Bostelmann (1962) may prove useful for future studies of the effects of intense noncavitating ultrasound on enzymes *in vivo*.

It is felt that the most promising future research into the nature of the interactions between intense noncavitating ultrasound and tissues will

deal with those levels of biological structure which lie between the molecular level and structures observable with the light microscope. Survival and genetic studies on populations of irradiated microorganisms are probably the best approaches to learn more about the effects of intense noncavitating ultrasound on DNA in vivo. A study of the effects of intense noncavitating ultrasound on the properties of membranes would be useful not only because membranes are a ubiquitous structural feature of cells, being found around the nucleus and as a part of such cellular organelles as mitochondria, endoplasmic reticulum, etc., but also because of the importance of excitable membranes in nervous tissue. When information from studies such as those just suggested becomes available, a correlation of all the information on all levels of structure will provide a better understanding of the effects of intense noncavitating ultrasound on tissue.

APPENDIX

CONSTRUCTION AND CALIBRATION OF THE ULTRASONIC IRRADIATION REACTION CELL INCORPORATED IN THE SPECTROPHOTOMETER

A Beckman DU spectrophotometer was modified to allow ultrasonic irradiation of a solution while simultaneously monitoring the amount of light of a selected wavelength absorbed by the solution. The modified spectrophotometer makes possible the study of ultrasonically induced changes in a static solution by observing changes in the absorption of visible and/or U.V. wavelengths, and also permits the observation of the effects of ultrasonic irradiation on the reaction rates of chemical reactions which can be monitored spectrophotometrically.

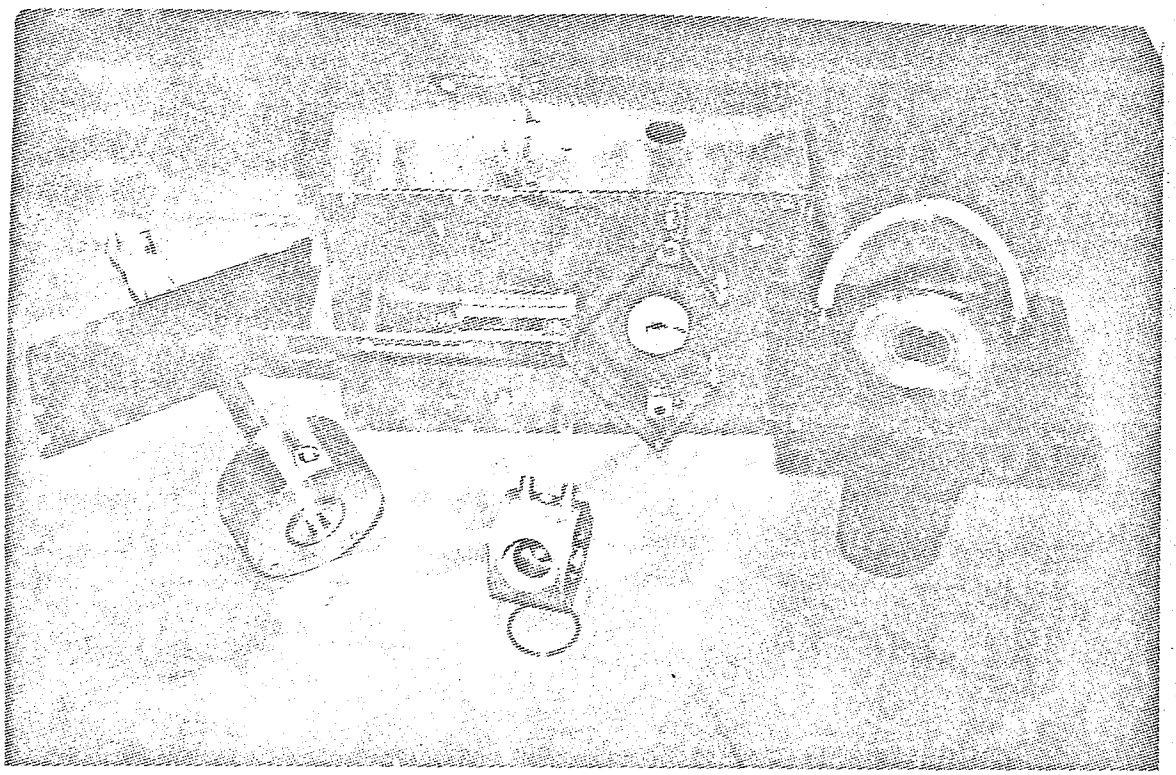
Monitoring the absorbance at 260 mμ of deoxyribonucleic acid (DNA) while it undergoes ultrasonic irradiation is an example of the use of this type of instrument with static solutions (Hawley et al., 1963). The observation of ultrasonically irradiated enzyme reactions is the subject of part of this report.

The principal modification of the spectrophotometer is in the sample compartment. The cuvette compartment was replaced by the larger compartment shown in Figures 3(b) and 15(b). A quartz collimating lens, made for use with a 25 cm special Beckman cell, was used in place of the usual slit lens. Light emerging from the slit can be sent on two alternate routes, through the blank cell or the irradiation sample cell, via two rotating and four fixed front surface mirrors as shown in Figure 16.

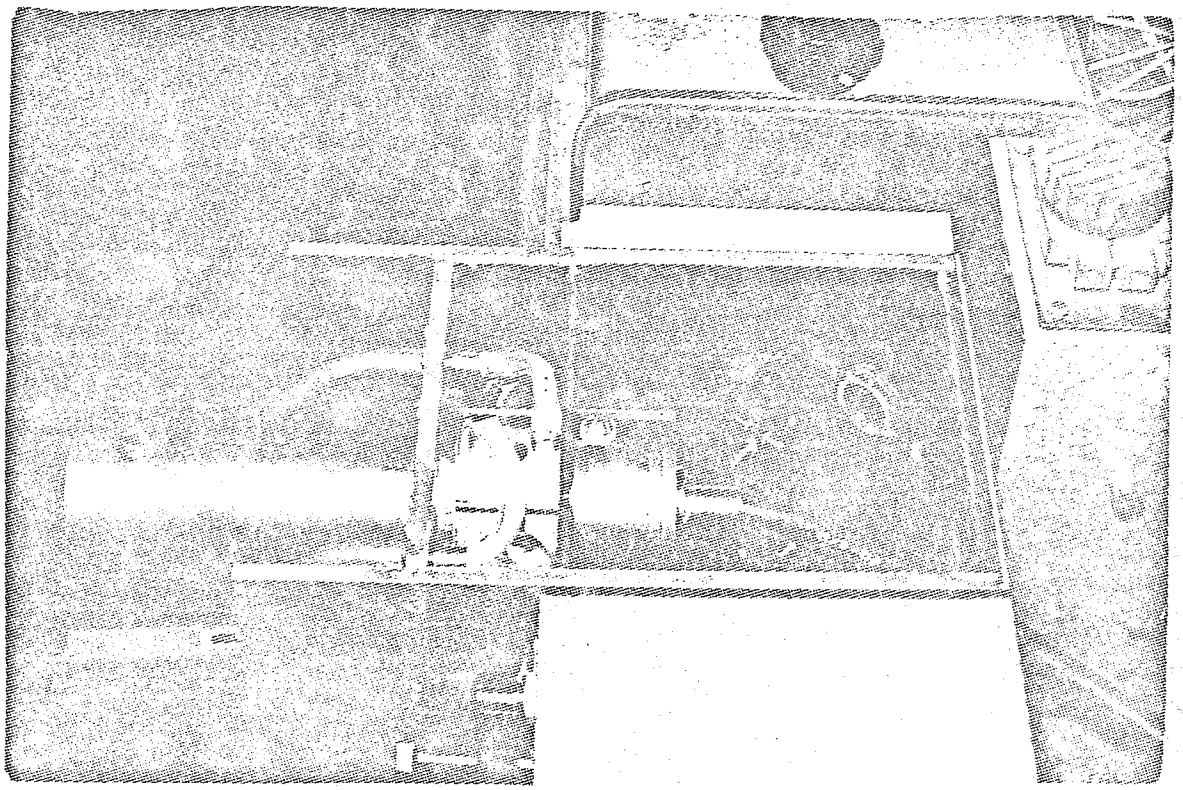
Optical Compartment

The housing which contains the dual optical paths, the blank cell and the irradiation sample cell was constructed of 1/4 inch aluminum plate except

FIGURE 15



a.) COMPONENTS OF THE REACTION IRRADIATION COMPARTMENT

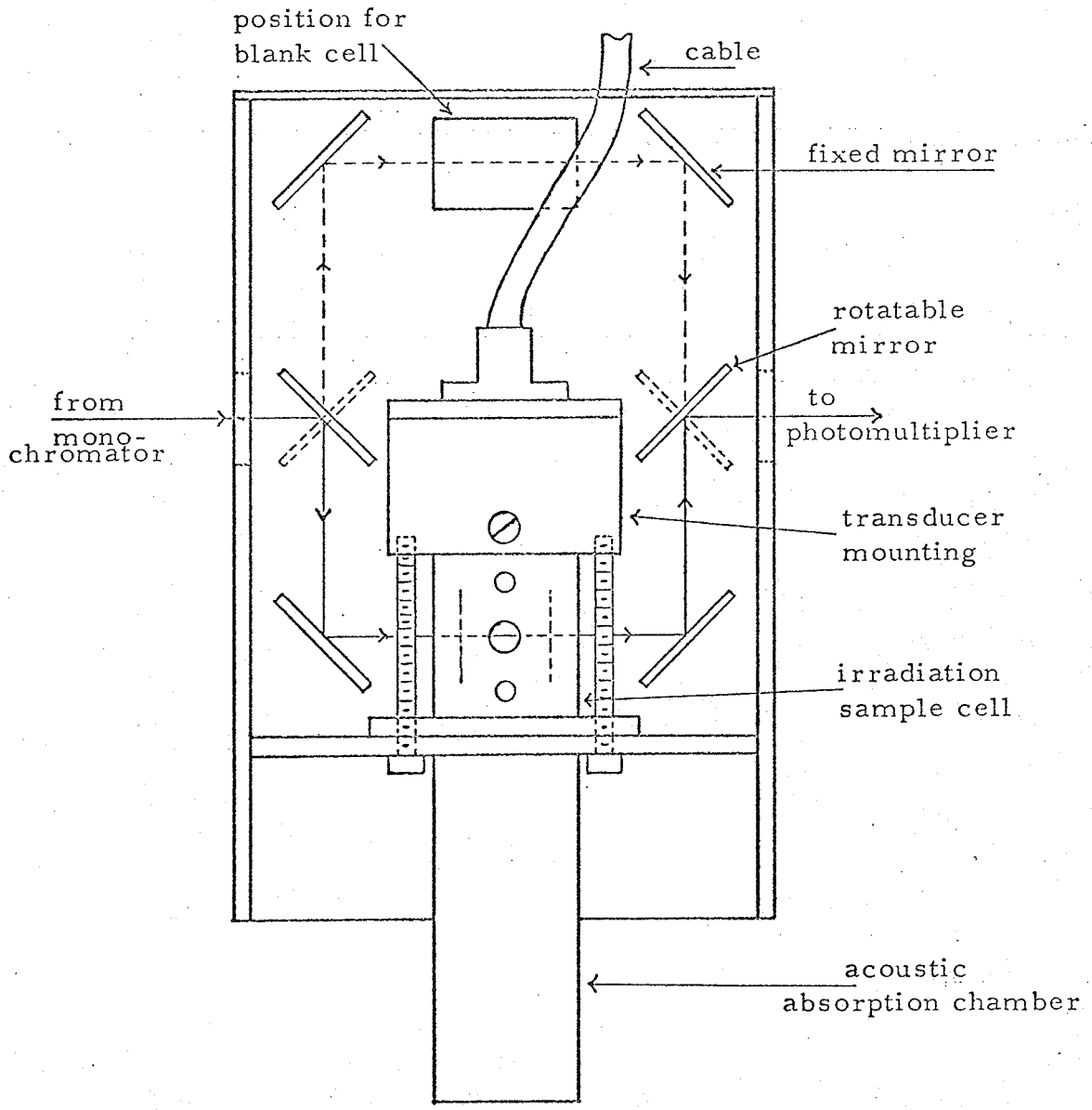


b.) COMPARTMENT IN SPECTROPHOTOMETER

FIGURE 16

OPTICAL PATHWAYS IN MODIFIED SPECTROPHOTOMETER SAMPLE COMPARTMENT

TOP VIEW



for the 1/16 inch thick aluminum back plate through which the cable from the electronic driver passed. The compartment's outside dimensions were 9 inches long by 6 inches wide by 3 1/2 inches high. The inside of the compartment was sprayed with flat black paint. The front plate served as a mounting bracket for the irradiation sample cell. Mounting is accomplished by passing the acoustic absorption chamber through the hole provided for it in the front plate until the flange at the open end of the absorption chamber is flush with the inside of the front plate, passing four 8/32 screws through the front plate and the flange into the threaded holes in the transducer housing, and clamping the irradiation sample cell between the transducer housing and front plate by tightening the four screws (Figures 15(b) and 16). The cell is rendered liquid tight by "O" ring seals between the cell and transducer housing at one end and the cell and absorption chamber flange at the other end. Also mounted on the front plate are two short lengths of copper tubing through which water from the constant temperature bath passes to and from the walls of the irradiation sample cell. The whole compartment is mounted in the spectrophotometer by means of four screws in the same way as the original cuvette compartment, only it is supported by longer screws.

Optical System

The four fixed and two rotatable mirrors were made by vacuum depositing aluminum on optical flats. The mirror mounts were made of brass and spray painted black. Each mirror mount includes three small adjusting screws for making fine adjustments in the mirror's position to align the optics. The two rotatable mirrors can be rotated simultaneously through 90^{o} to shift the light beam from the blank cell to the irradiation sample cell and vice versa. The simultaneous shift is accomplished by connecting two spring loaded levers to a single bar arranged to act as a two-position switch (Figure 15(a)).

Ultrasonic Irradiation System

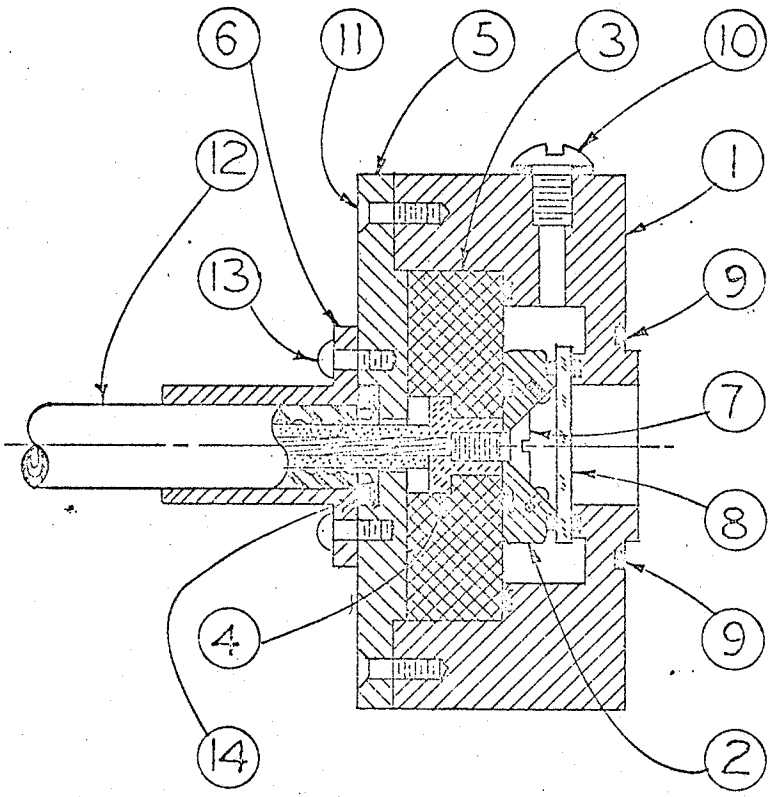
The ultrasonic irradiation system consists of three components: the piezoelectric transducer to convert the electrical energy to ultrasound; the irradiation cell which contains the sample, maintains it at constant temperature, and which possesses windows to transmit the spectrophotometer light beam; and the acoustic absorption chamber absorbing all incident acoustic energy. The construction of each component is considered separately.

Two transducer housings were constructed, to mount X-cut quartz plates with fundamental resonant frequencies of 1 and 9 MHz. The polished quartz plates, each one inch in diameter, were obtained from the Valpey Corporation. Gold over chromium electrodes were vacuum deposited on the plate faces. The mounting of the transducer in its housing is illustrated for the case of the 1 MHz crystal in Figure 17. The material for the various parts shown is No. 303 stainless steel except as noted on the figure. The face in direct contact with the sample solution is grounded via the transducer housing to the shielding of the RG 8/u coaxial cable. Gold foil, 0.001 inches thick (not shown in the diagram), covers the face of the back-up plate to provide a gold-to-gold contact for the high-potential connection to the back of the transducer. The space in the transducer housing surrounding the transducer and back-up plate is filled with degassed Dow Corning 200 Silicon fluid to provide a high dielectric strength medium between the two transducer electrodes. The high dielectric strength is especially important if high voltages are to be developed across the electrode of the 9 MHz transducer since it is only 0.013 inches thick.

The irradiation sample cell is shown to scale in Figure 18(a). Except for silver solder and the quartz windows, the cell is constructed entirely of

CROSS SECTION OF TRANSDUCER HOUSING

1. transducer housing
2. back-up plate
3. insulator (epoxy resin)
4. cable connector plug (brass)
5. pressure plate
6. cable shield clamping plate
7. 8-32X1/4" connector screw
8. 1MHz X-cut quartz crystal, gold-on-chromium vacuum deposited electrodes
9. "O" ring (neoprene)
10. 12-24X3/8" screw, oil filling hole closure
11. 6-32X1/2" screw
12. RG 8/U coaxial cable
13. 3-48X3/8" screw
14. gasket (neoprene)



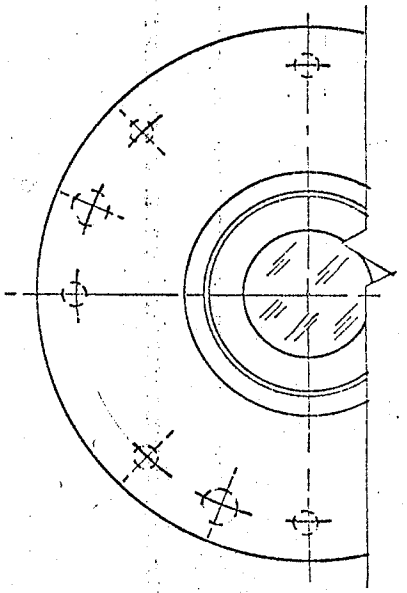
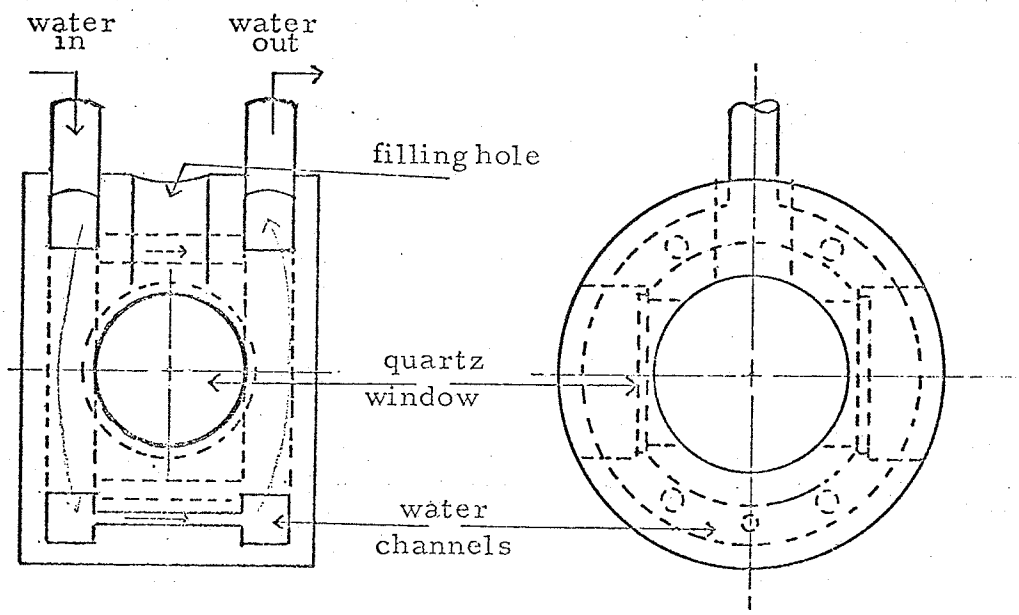
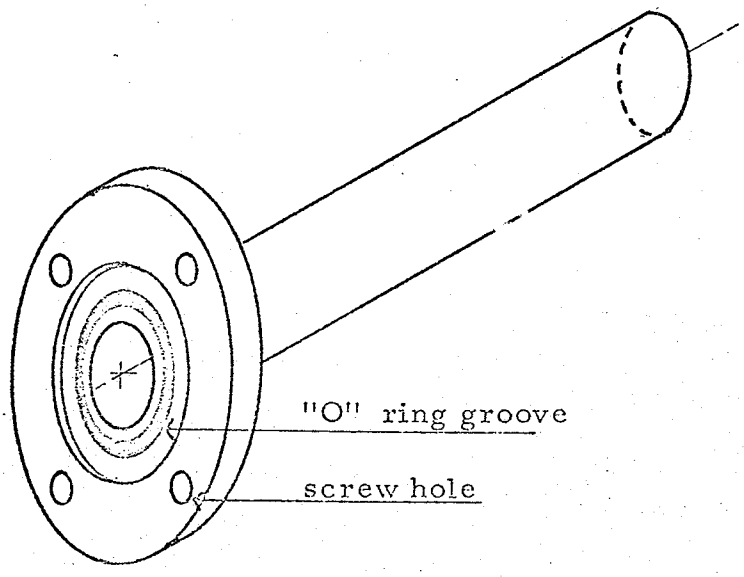


FIGURE 18



a.) TEMPERATURE CONTROLLED IRRADIATION SAMPLE CELL



b.) ACOUSTIC ABSORPTION CHAMBER

No. 303 stainless steel. The 1 mm thick quartz windows were cemented in place with epoxy resin. The volume of the cell at 25.0°C, to the shoulder of the filling hole is 21.6 cm^3. The length of the light path in the sample solution is 2.870 cm. By arranging the holes which connect the two annular water channels so that holes of larger diameter are at the top of the cell and so that the total cross section of all the holes is slightly less than the cross section of the pipe leading into the cell walls, a uniform flow of water is maintained within the walls of the irradiation sample cell. Connections between the intake and outflow ports of the sample cell and the copper tubes passing through the front plate of the housing are made with "Tygon" tubing. The sample cell is separated from the castor oil absorption chamber by a 0.001 inch polyethylene membrane.

The acoustic absorption chamber (Figures 15(a) and 18(b)) is a brass cylinder, two inches in diameter, six inches long, closed at the far end and soldered to a circular brass flange, three inches in diameter and 1/4 inch thick at the near end. The opening in the center of the flange into the cylinder is 7/8 inch in diameter and is surrounded by a 1/16 inch lip which fits into the one-inch bore of the irradiation sample cell. A two-inch diameter depression 0.062 inches deep and an "O" ring groove were turned in the face of the flange surrounding the opening to provide a snug fit and liquid-tight seal when the irradiation sample cell and absorption chamber are compressed together in mounting.

Characterization of the Ultrasonic Field Within the Irradiation Sample Cell

A 0.625 inch diameter area of the plated transducer surface is in direct contact with the sample solution and radiates ultrasound down the length of the one-inch diameter cylindrical sample cell volume. The intensity of the sound beam and the distribution of the intensity within the sample cell were

determined by the radiation pressure method (Fry and Dunn, 1962e) which measures the effect of the unidirectional force due to radiation pressure on a small stainless steel sphere. For these measurements, a stainless steel sphere, 0.062 inches in diameter, was attached to a nylon monofilament and lowered down a 10 cm standpipe mounted above the sample cell's filling hole into the sample cell. The sphere was positioned within the sample cell by means of a mechanical three-dimensional coordinate system. The deflections of the sphere were observed through the quartz side windows with a cathetometer which could be ready directly to the nearest 0.001 cm. Since the deflection of the sphere is directly proportioned to the ultrasonic intensity, the distribution of the intensity within the cell could be determined by moving the sphere about in the sample cell and observing the change in deflection of the sphere.

Results of the calibration in the center of the sample cell for one and nine megacycle transducers are given in Figures 19 and 20. The intensity is uniform along the axis of the cylindrical sample cell, the direction of ultrasound propagation. Vertically and laterally from the cell's axis the intensity drops off gradually to approximately two-thirds of its peak axial value at the edge of the 0.625 inch diameter beam and more rapidly beyond this point. Calibration curves for the one-megacycle transducer being driven at nine megacycles and for the nine-megacycle transducer being driven at twenty-seven megacycles are Figures 21 and 22, respectively.

The frequency of the ultrasound within the sample cell was checked, particularly when operating on harmonics of the fundamental resonant frequency, by means of a small (1 mm diameter, 3 mm long) barium titanate piezoelectric probe by displaying the signal from this detector on an oscilloscope.

FIGURE 19

INTENSITY VERSUS VOLTAGE SQUARED RELATIONSHIP AT 1MHz
FOR 1MHz QUARTZ TRANSDUCER

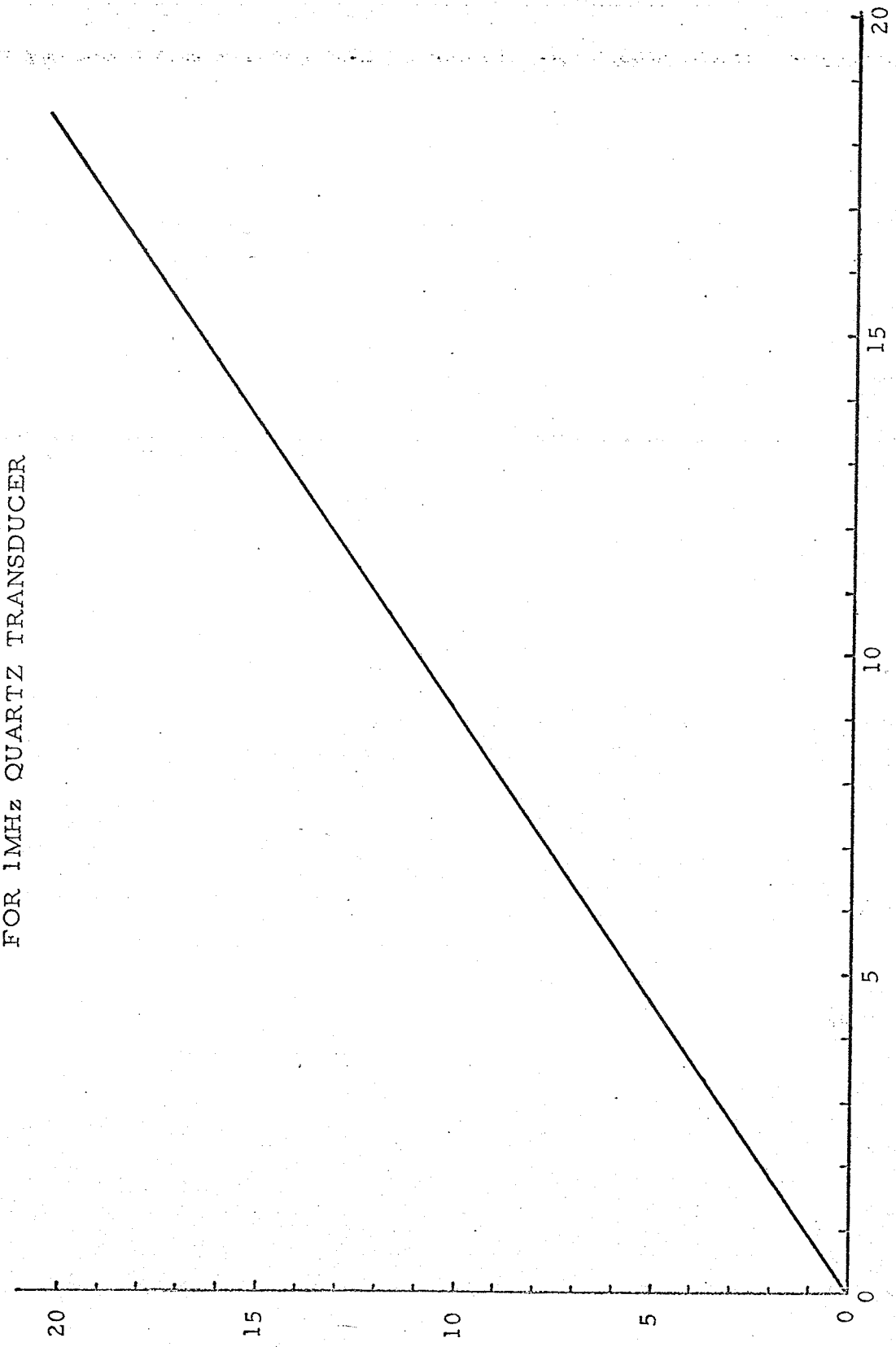


FIGURE 20

INTENSITY VERSUS VOLTAGE SQUARED RELATIONSHIP AT 9MHz

FOR 9MHz QUARTZ TRANSDUCER

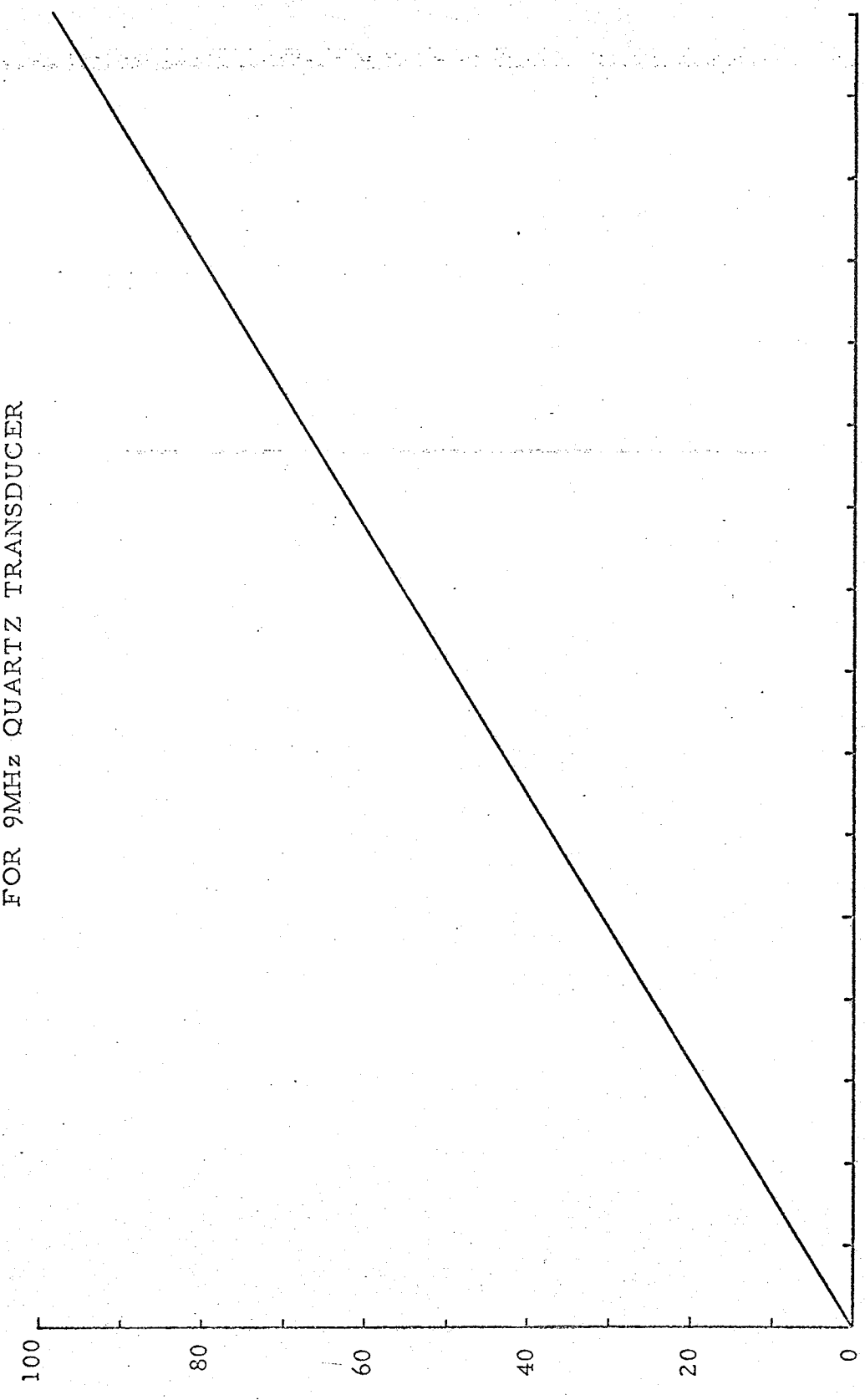


FIGURE 21

INTENSITY VERSUS VOLTAGE SQUARED RELATIONSHIP AT 9MHz
FOR 1MHz QUARTZ TRANSDUCER

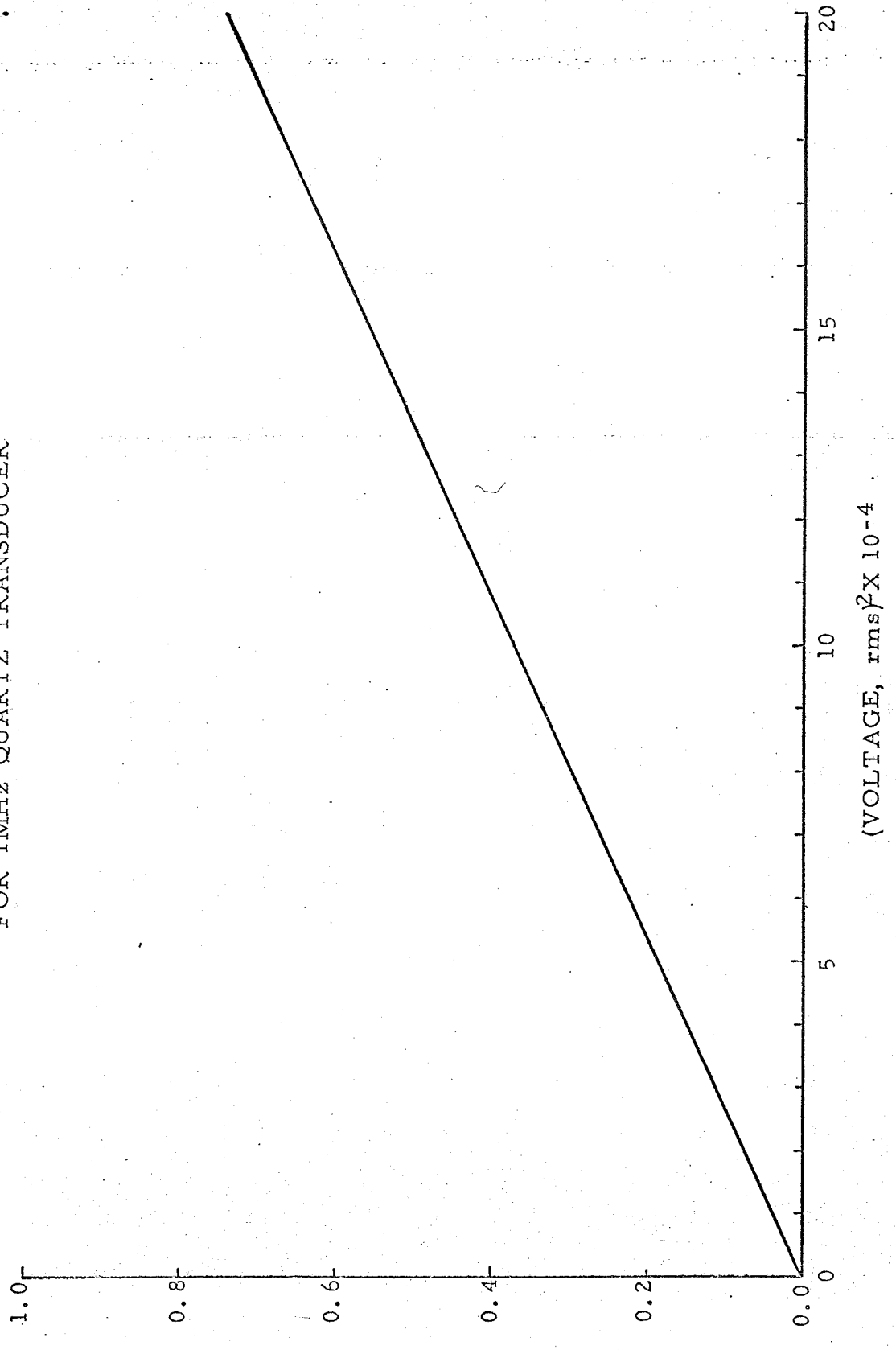
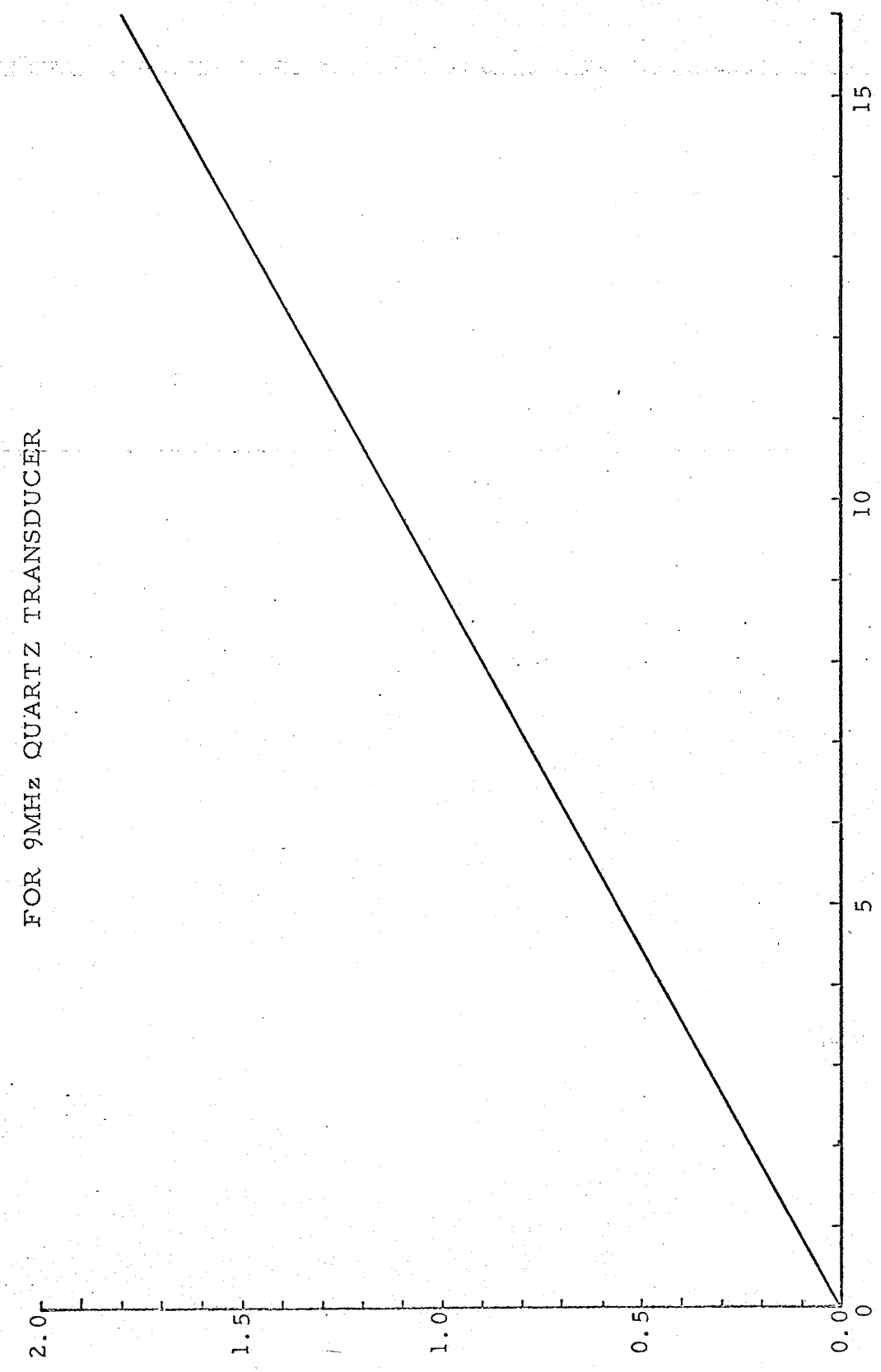


FIGURE 22

INTENSITY VERSUS VOLTAGE SQUARED RELATIONSHIP AT 27 MHz

FOR 9MHz QUARTZ TRANSDUCER



Methods used for detecting cavitation in an irradiation sample cell of this type have been described previously (Hawley et al., 1963).

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VITA

Robert Malcolm Macleod was born in New York City on December 7, 1935. He was raised and received his primary and secondary education in Kalamazoo, Michigan. Upon graduation, cum laude, from Kalamazoo Central High School in 1953 he received a Science Scholarship to attend Kalamazoo College. After graduating, cum laude, from Kalamazoo College in 1957 with majors in Mathematics and Chemistry, he accepted a State College Scholarship to do graduate work in Mathematics at the University of Michigan. He left the University of Michigan in August of 1958 and began two years of military service in the Chemical Corps of the U. S. Army in December of 1958. Upon completion of active military service he entered the University of Illinois as a graduate student in the Biophysical Chemistry Program in February of 1961. He received his M.S. with a major in Biochemistry in June of 1962 and accepted a National Institute of Health Traineeship to continue graduate work toward a Ph.D. degree in Biophysics. In September of 1964 he became a Research Assistant at the Biophysical Research Laboratory at the University of Illinois and remained in this position until completion of his Ph.D. thesis in September of 1966.